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Litton Bionetics, Inc., Kensington, MD

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AEERL PROCEDURES MANUAL:
LEVEL 1 ENVIRONMENTAL ASSESSMENT
TERRESTRIAL ECOLOGICAL TESTS

by

Thomas A. Gezo
(Union Carbide Corporation)

and

David J. Brusick
Litton Bionetics, Inc.
Kensington, MD 20895

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Raymond G. Merrill
Technical Support Office
Air and Energy Engineering Research Laboratory
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Research Triangle Park, NC 27711

AIR AND ENERGY ENGINEERING RESEARCH LABORATORY
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Ecology	Environmental Assessment	06F
Bioassay		06A
Cultures (Biology)	Terrestrial Ecology	06C, 06M
Ethylene	Plant Stress Ethylene	07C
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ABSTRACT

Some test methods designated for the AEERL Level 1 environmental assessment biological testing program are sufficiently new that little or no published literature is available describing specific application of the procedures. This manual provides detailed procedures for the AEERL Level 1 terrestrial bioassays. The manual is designed to supplement the "IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests" (Second Edition) (EPA-600/8-81-024, NTIS PB 82-228966) which provides the basic procedural steps. The protocols described in this document are the same as those in the second edition of the manual, but are expanded in those areas of methodology which involve culturing methods, materials and supplies, and specific scoring procedures.

The three tests included in this document are: (a) Plant Stress Ethylene Test, designed to assess the toxic effects of gaseous samples on plant foliage; (b) Root Elongation Test, to determine the toxic effects of liquid samples and aqueous leachates on seed germination and root elongation in a number of plant species; and (c) Insect Toxicity Test, used to measure lethality and reproductive toxicity in Drosophila melanogaster.

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FOREWORD

This report was prepared for EPA to complement the general guidance for application of terrestrial biological tests to engineering research and development projects for the Air and Energy Engineering Research Laboratory. This report provides specific procedural information not found in journal literature or other EPA publications. The manual is part of a series of procedures documents describing the phase approach for environmental assessment. The primary focus of the test methods is investigation and screening of the potential biological effects of stationary (point) source emissions and the effect of various control technologies on those emissions. The goal of such an approach is to provide guidance for research purposes used in the development of engineering control technology.

Application of these test methods should be done to screen unknown stationary source and process samples to rank effluents in a nonregulatory mode. The testing should be performed and interpreted by a Laboratory qualified to do terrestrial plant and insect testing and familiar with these tests. Evaluation of assays resulting from tests conducted on complex mixtures must be performed with all pertinent information including: source operation conditions, sampling parameters, relevant chemical analyses, and bioassay results. The conclusions generated from such tests are intended to guide research efforts in the choice of definitive or applicable regulatory methods.

CHAPTER 1

LEVEL 1 PLANT STRESS ETHYLENE BIOASSAY

by

Thomas A. Gezo
Union Carbide Corporation, Environmental Services
Tarrytown, NY 10591

SUMMARY

This section describes the Level 1 Plant Stress Ethylene (PSE) Test which is designed to assess and rank the phytotoxicity of gaseous effluents. The test employs the stress ethylene response of plants which is characterized by the increased production and release of ethylene in proportional response to physical and chemical stresses. Bush bean plants are exposed to three concentrations of test gas using a specially designed chamber. The plants are then enclosed in plastic bags are are incubated to allow ethylene to accumulate. Finally, ethylene is analyzed by gas chromatography and the plants are examined for foliar injury. In addition to experimental procedures the report describes chamber construction, gas sampling, plant culturing, interpretation of results, and the ranking of test material toxicity.

1.1 INTRODUCTION

The EPA Level 1 Plant Stress Ethylene test is designed to assess the toxic effects of gaseous effluents on plants by employing the stress ethylene plant response. Under normal conditions plants release low levels of ethylene which function hormonally to regulate growth and development. In response to various stresses, including chemical (1-7), light (8), water (9-12), temperature (13, 14), disease, and mechnical effects (5, 15, 16), ethylene production increases substantially (17). Viable plant tissue is necessary for ethylene production. Ethane evolution may also increase in response to some stresses and is thought to indicate more severe damage and tissue autolysis (3, 4, 18). Current evidence suggests that ethylene is a product of methionine metabolism and the same biochemical pathways are involved in the production of basal and stress-induced ethylene (1, 19). Ethane may be produced by peroxidation of linolenic acid upon extensive wounding and this peroxidation may also result in some ethylene production (19). Critical to the effectiveness and applicability of the stress ethylene test is that induction of stress ethylene is proportional to the intensity and duration of the stress over a wide range of stresses (16). However, the stress ethylene response has yet to be documented for a large number of pollutants and complex mixtures.

The test procedure has been designed to minimize laboratory manipulation in order to provide a cost effective bioassay. The procedure involves culturing of test plants, gas sampling, exposure, incubation, ethylene analysis, and visual examination. Bush bean, Phaseolus vulgaris L.,

cultivar Harvester, has been selected as the bioassay plant because it develops rapidly and is easy to grow. The plants are cultured under controlled conditions and are subsequently exposed to the test gas. After a period of dark incubation in sealed containers, ethylene and ethane are analyzed by gas chromatography. Three days after assay the plants are examined visually for injury.

1.2 GAS SAMPLING

Gas samples are collected in 150-liter polyvinyl fluoride film bags (Tedlar* or equivalent, 2 mil [50.8 μm] thick, 36 x 50 inches [91.4 x 127 cm]). The bags are heat-sealed closed with 1-inch seams, cleaned, and leak tested before use. Tedlar bags may be purchased commercially or made in the laboratory. The bags are cleaned with a solution of dichloromethane and methanol (1:1, v/v) and are dried by purging with nitrogen. To facilitate handling and to keep the gas samples in darkness, the bags are contained within 55-gallon (208.2-liter) fiber drums. Access to the sample bag is through a 1/2-inch (12.7-mm) polypropylene gate valve attached to the bag by means of a polypropylene tank fitting and to the metal drum lid with a PVC coupling (Figure 1.1). The gate valve lies within the fiber drum to avoid damage during shipment. To prepare a bag for sampling, it is evacuated, closed via the gate valve, and attached to the drum's lid.

Sample bags are filled individually during sampling using an additional gate valve attached to the outside of the drum to control the rate at which the bag is filled. The external valve is attached to the stack sample port with Teflon tubing. A smaller opening on the opposite side of the lid is to be used when sampling streams with negative pressure. A high-speed blower is attached to this port and used to lower the pressure in the drum below that of the sample stream. The sample stream can then fill the evacuated Tedlar bag without having to be pumped. During sampling, pressure within the bag is monitored by attaching one arm of a U-tube manometer to the sampling line near the drum's sample port and leaving the other arm open to the atmosphere. This allows the filling procedure to be monitored and helps prevent bags from bursting. A view port cut in the top of the drum and sealed with plastic is also useful in this respect.

After collection of the sample, the drums are promptly shipped to the testing laboratory. If the sample can be tested within three days, it may be stored at room temperature. If a longer period is necessary, the sample should be stored at +4°C and permitted to warm to the temperature used in the test prior to test initiation. Bags may be reused several times. After the sample is exhaused, the bag is cleaned as previously described. The cleaned bag should be leak tested before reuse.

*E.I. Dupont de Nemours and Co. (Inc.), Wilmington, DE 19898.

Figure 1.1 Sample Container LID With Sample Bag Attached

1.3 EXPOSURE CHAMBER DESIGN AND CONSTRUCTION

The exposure chambers (Figures 1.2 and 1.3) used for this test are similar to those described by Heck et. al. (20). A negative-pressure single-pass flow system is used to draw gas into the chambers. The chambers are then closed to allow static exposure of the plants to the test gas. Three chambers are required for the test gas and additional chambers are required for positive and negative controls.

The chambers are 24 inches (61 cm) wide by 22 inches (55.9 cm) deep and 30 inches (76.2 cm) high with a 3/4-inch (19.05-mm) plywood frame. Mylar film (1 mil, 25.4 μm) is attached to the frame with duct tape to form the sides and top. A 1/2-inch (12.7-mm) plywood bottom is used and a false bottom of 1/4-inch (6.35-mm) pegboard is placed 6 inches (15.24 cm) from the chamber floor. The door is positioned in the frame using four wooden strips with a centered clamp on each and 3/8-inch (9.53-mm) Tygon tubing is used for a seal. All wooden components are painted with epoxy paint to minimize gas absorption and allow thorough cleaning of the chambers.

An internal air circulation system is added to each chamber to provide air circulation during exposure and to prevent concentration gradients from developing within the chamber. A small blower is mounted below the false bottom and is connected to a vertically mounted 1-1/4 inch (31.75 mm) diameter polypropylene pipe. The blower draws in air from beneath the false bottom and exhausts it through the pipe into the upper area of the chamber. The motor driving the blower is mounted outside the chamber to prevent ozone and hydrocarbons (volatilized lubricants) from entering the chamber and influencing the test results.

Each of the chambers is attached to the test gas intake and exhaust manifolds (Figure 1.4). A gas container manifold consisting of a 1-1/4 inch (31.75 mm) diameter polypropylene pipe closed at one end and a 1-1/4 inch (31.75 mm) polyethylene valve at the other end is used to conduct the test gas from the sample containers to the intake manifold. Provisions are made along the manifold for attaching ten 55-gallon (208.2-liter) sample containers. The intake for each chamber consists of a 1-1/4 inch (31.75 mm) diameter pipe and an on-off ball valve connected to the intake manifold. The intake manifold terminates with a junction permitting attachment of the gas sample container manifold. The chamber exhaust system uses 3/4-inch (19.05-mm) polypropylene pipe and includes an on-off ball valve, the orifice-type calibrated flow meter (using a water manometer across the orifice to measure the pressure drop), and a regulating valve. This system is connected to the exhaust manifold. A high pressure 20,000 rpm blower (Cadillac or equivalent) is used in the exhaust system to draw the sample being tested into the chamber. Inlet tubing and fittings are made of polypropylene to reduce corrosion and contamination; exhaust fittings are made of polypropylene, Tygon and PVC. The operating characteristics of this system are similar to those reported by Heck et. al. (20) with a flow of 142 liters/min (4 cu ft/min) and a negative pressure of 3.8 mm H_2O measured in the chamber.

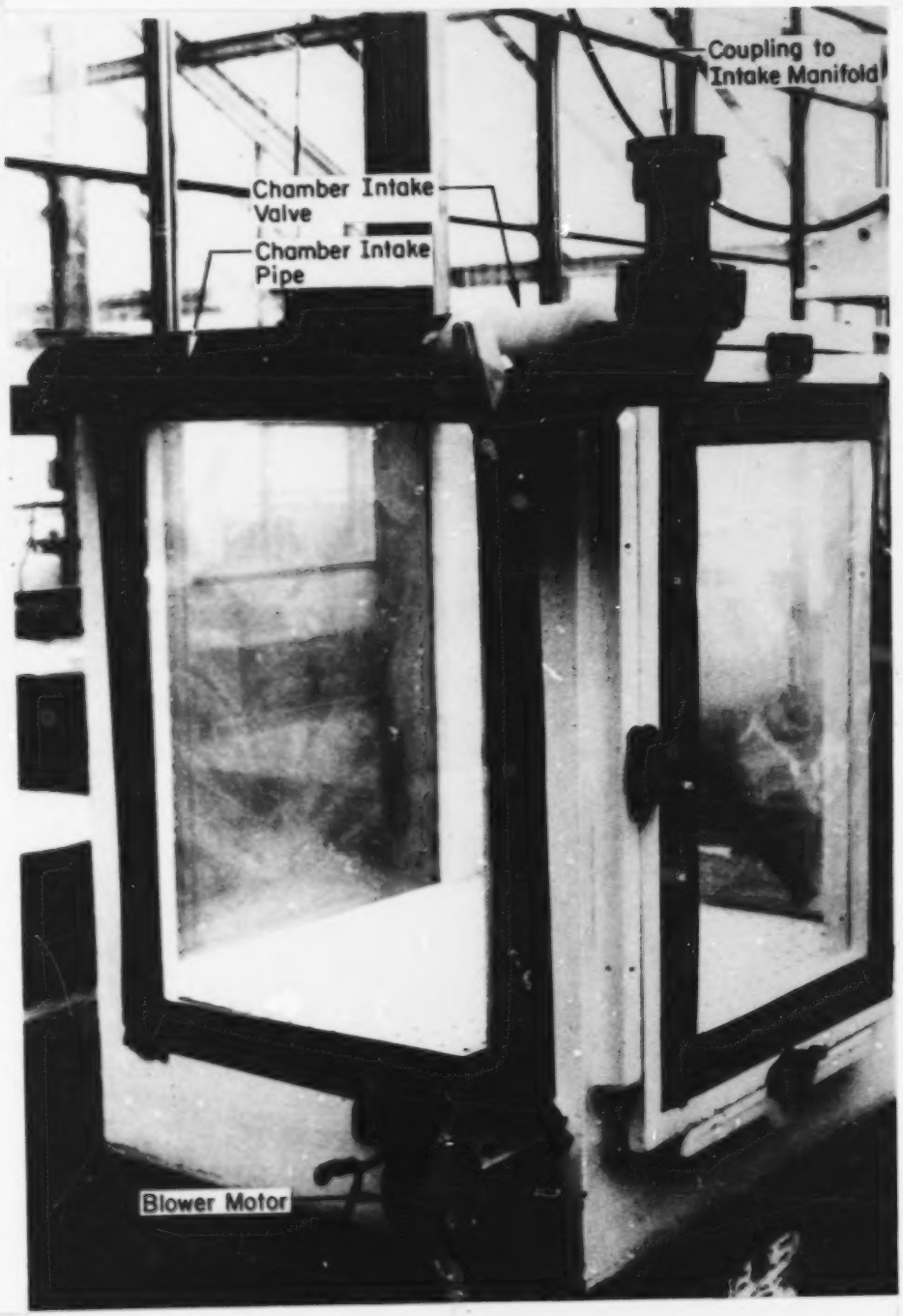

Figure 1.2 Intake (Left) Side of the Exposure Chamber

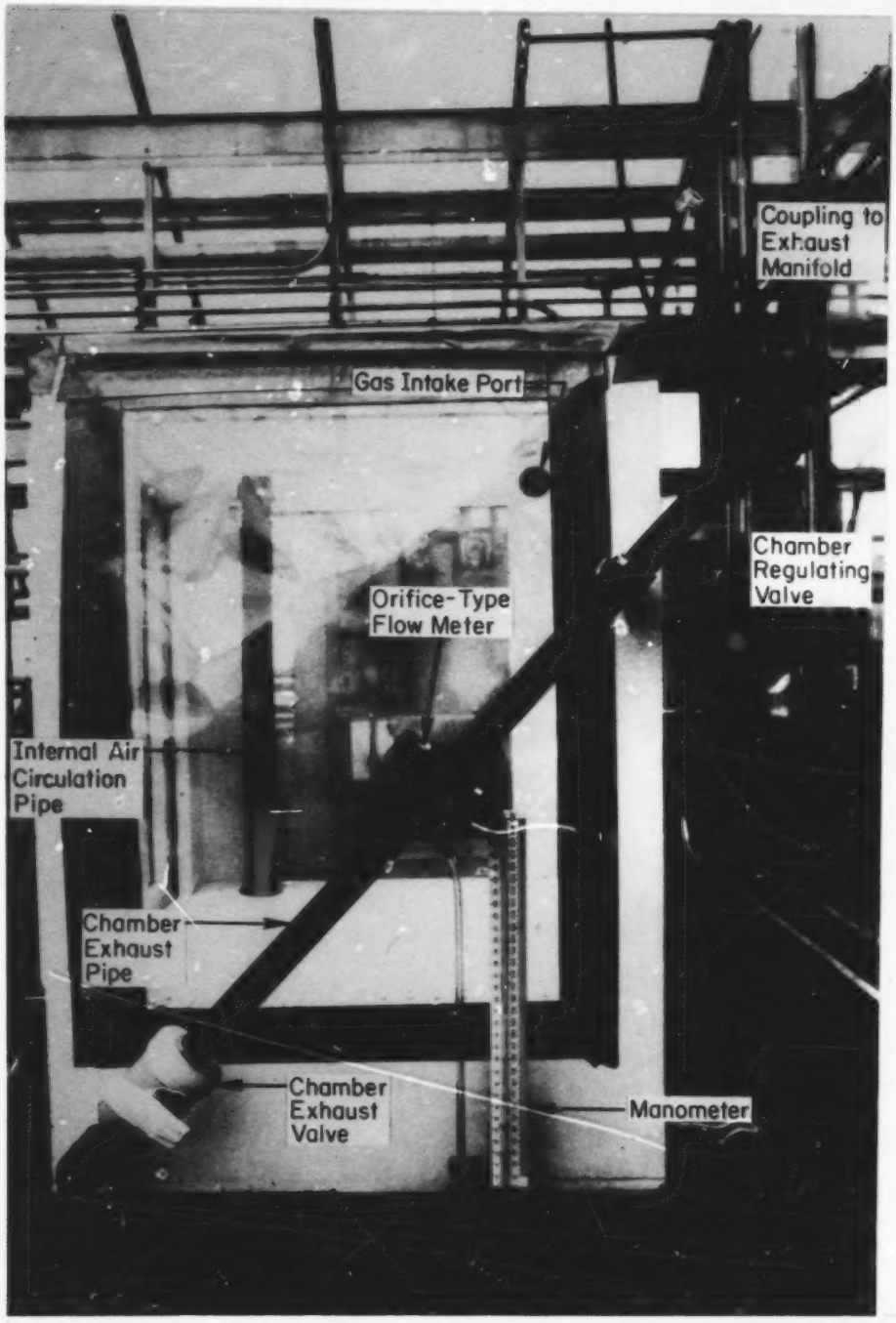

Figure 1.3 Exhaust (Right) Side of the Exposure Chamber

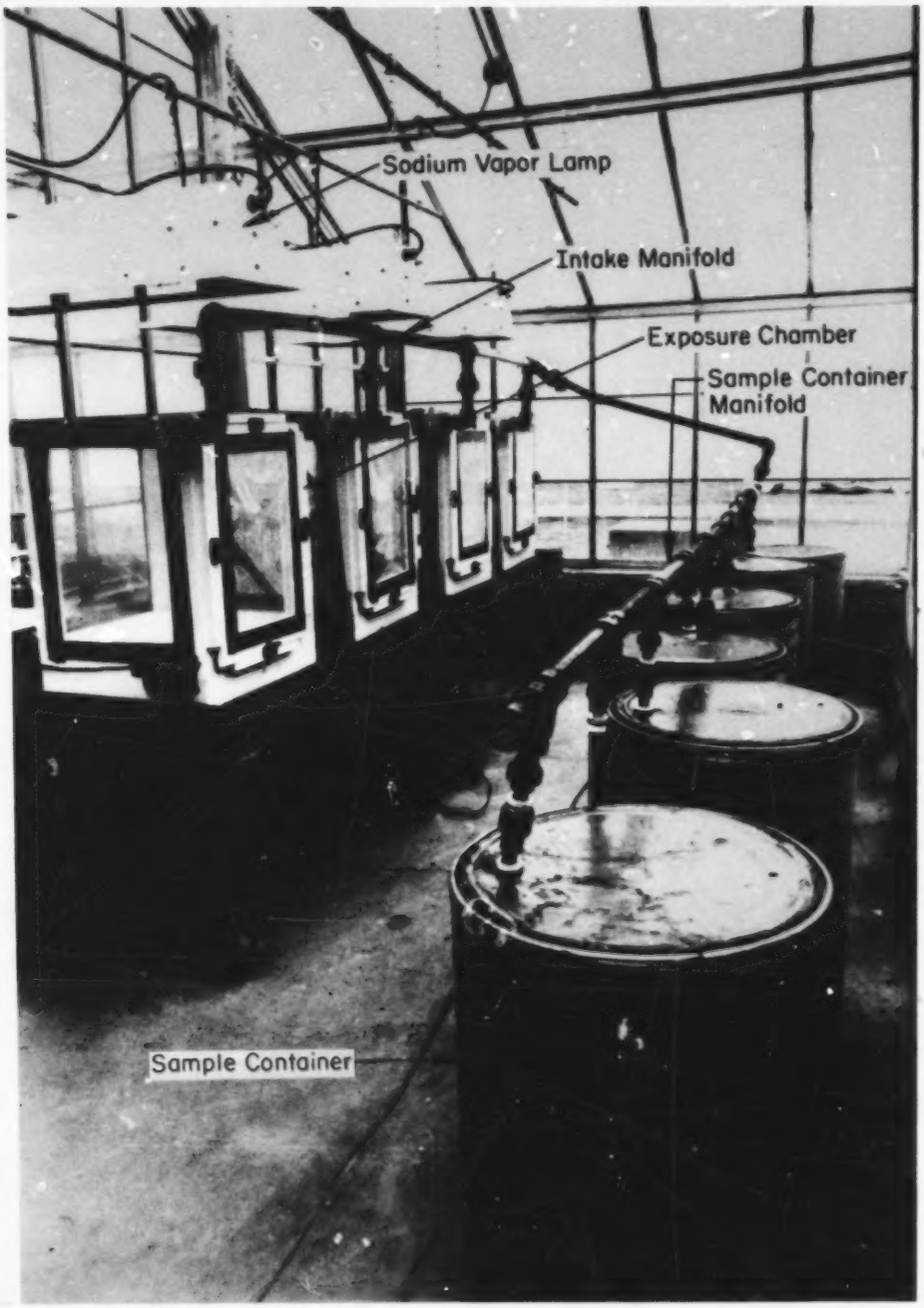

Figure 1.4 Complete Stress Ethylene Exposure Set-up Showing The Various Components and Their Interconnections (only 6 of the 10 sample containers are shown here)

1.4 PLANT CULTURING

Bush beans, Phaseolus vulgaris L., cultivar Harvester, are grown from seed for use in the Plant Stress Ethylene Test. A total of 8 plants are required for each test gas concentration and each control. It is required that 12 be grown for each condition so that uniform plants may be selected. Two seeds are planted in 10-cm (4-inch) plastic pots using pro-mix BX or Jiffy Mix as the potting medium. The seedlings are thinned to one seedling per pot 7-8 days after seeding. Plants are surface watered daily with sufficient half-strength Hoagland's nutrient solution (21) to require the excess solution to drain. Temperature is monitored at plant level using a shielded temperature detector and plants are maintained at average day/night temperatures of 26°C and 20°C respectively. A photoperiod of 16 hours light and 18 hours dark is recommended. During the photoperiod the light intensity must be a minimum of 100 g cal/cm^2/day (400-700 nm) as measured at plant height. An average light intensity of 200 g cal/cm^2/day is preferred. When bush beans are grown in a controlled environment chamber, air is continuously renewed to prevent CO_2 depletion.

The plastochron index (22,23) is used to select plants for assay to ensure that plants are exposed at a standard time of development and to ensure population uniformity. Bush beans are exposed within 24 hours of the time when the population being treated exhibits a plastochron index of 1.5 ± 0.3 (24). The plastochron index (22,23) is computed using a random sample of ten plants and 20 mm as the control leaf length. The cotyledonary and primary leaves are excluded from the leaf count in the determination of plastochron index. In the selection of assay plants, non-uniform plants are to be avoided. The growth period ranges from 12 to 21 days depending upon the exact environmental conditions.

1.5. EXPERIMENTAL PROCEDURE

1.5.1 Exposure

Bush beans are exposed to three concentrations of test gas in the test chambers, a fourth chamber with charcoal filtered air is used as a negative control and an additional chamber is used for the positive control (chlorine gas). The exposure is carried out at room temperature and lighting is provided by 400 watt high pressure sodium vapor light (or the equivalent). Eight plants are used for each exposure level and are placed into the chamber carefully to prevent jarring, being sure that adjacent plants do not touch or shade one another. The exposure is carried out sequentially with the negative control chamber being run first, followed by the experimental chambers in order from the high to low test gas concentration and then positive controls. The exposures to all test gas concentrations are started within one hour of the start of the negative control.

The objective is to have each chamber contain twice as much test gas as the chamber with the next lower concentration. The mixing curve for the test gas in the chamber air is logarithmic. Thus, to double the concentration x which results from a mixing time of y would require doubling the mixing time (2y). To double the concentration again (4x) would require

eight times the mixing time (8x). The actual mixing time for any set of chambers will be a function of the rate of flow of gas through the chamber and the total quantity of test gas available. The mixing curve for any chamber design should be empirically determined using an easily detectable reference gas. Methane at 80 parts-per-million (ppm) may be used and the change in chamber concentration measured with a hydrocarbon analyzer.

The flow rate for each chamber must be calibrated prior to exposure. A rotameter is placed in the exhaust system between the blower and the chamber and used to calibrate the orifice-type flowmeter of each chamber. A flow of 5 cfm (142 l/min) is established using the rotameter for reference and the reading in cm of water on the manometer connected across the orifice is recorded. The rotameter is subsequently removed and the flow adjusted with the regulating valve to achieve the previous reading on the manometer, completing the calibration. The sample container manifold is attached to the ten gas-sample containers, the valve on the manifold is closed and the valves on the individual containers are opened. Eight plants are placed in each chamber and the internal blowers activated. The negative control chamber is filled first. The external blower is turned on, the valves on the control chamber opened, and charcoal filtered room air is drawn through the chamber from the open intake manifold for two minutes. (The regulating valve may need to be adjusted slightly to ensure a 5 cfm flow). The chamber is then closed and permitted to incubate for two hours under a 400-watt high-pressure sodium-vapor light at a temperature of between 22 and 28°C. This step is designed to enable the plants to actively photosynthesize and metabolize to take up the test gas. Positive control chambers are filled similarly with filtered room air. Sufficient chlorine gas, calculated to give a chamber concentration of 15 ppm, is injected directly into the chamber.

Filling of the experimental chambers is conducted analogously. The sample container manifold is connected to the intake manifold and its valve opened. The valves on the first experimental chamber are opened and the test gas is drawn through the chamber for seven minutes. The chamber is closed and incubated under a 400-watt high-pressure sodium-vapor light for two hours. The remaining two experimental groups are exposed in the same fashion, except that the test gas is drawn in for two minutes and one minute, respectively. Exposure times were developed from the equilibrium curve presented by Heck et al. (20) which shows that the concentration of gas doubles from one minute to two minutes of mixing and again from two to seven minutes. After two hours of exposure to the test gas, the contents of each chamber are exhausted through a charcoal filter and the chambers are opened. The exposure incubation period allows maximum exposure of the bush beans to the gas while minimizing CO_2 depletion in the chamber.

1.5.2 Incubation

Immediately after exposure the test plants are examined for injury, are enclosed individually in plastic bags and are incubated post-treatment in the dark to allow ethylene accumulation. Plants are carefully removed from the chambers and are visually inspected for signs of damage, noting obvious effects such as lesions, wilting, epinasty, or discoloration. The plants

are then individually sealed within B-620 bags* (Cryovac; 10 inch x 32 inch [25.4 cm x 81.28 cm]; std gauge) with a wire support (10-gauge galvanized wire) surrounding the plant to prevent the leaves from touching the bag (Figures 1.5 and 1.6). To enclose the plant the bag is placed on a flat surface with its sides rolled, the plant is then placed on the bag bottom, and the wire support is positioned around the plant. Finally, the bag is drawn up and over the plant and is sealed using a wire tie. The bag should be sealed at a standard position so as to provide a constant volume for ethylene accumulation. Jarring may trigger the stress-ethylene response so care must be exercised during this step. Bagging is done on a laboratory cart so that further handling of the enclosed plants is minimized. The enclosed plants are incubated post-treatment at 26°C in the dark for at least two hours while ethylene accumulates within the bag.

1.5.3 Ethylene and Ethane Analysis

Ethylene and ethane are quantified by gas chromatography using a flame ionizaton detector (7). A Porpak-N column (2.4 m x 3.2 mm stainless steel, 80/100 mesh) is used for the separation and is operated isothermally at 80°C. The injection port and detector are maintained at 200°C and helium (40 ml/min) is used as carrier gas. Maximum sensitivity of the instrument is required as the limit of detection should be 5 parts-per-billion.† The GC is calibrated on each day that samples are to be analyzed using a minimum of five ethylene standards ranging from 100-1000 parts-per-billion to ensure linearity of response. (The response for ethane is assumed to be equivalent to the detector response for ethylene. Peaks are identified by retention time compared to standards and may be further documented by cochromatography with standard ethylene and ethane. A 1-ml sample is withdrawn from the bag surrounding the plant using a 1-ml gas tight syringe. The needle is inserted through the bag, a sample is withdrawn and analyzed by GC. The ethylene/ethane analysis requires approximately two minutes per sample. If ethylene concentrations for control plants exceed 50 parts-per-billion, improper plant handling is indicated and the test should be repeated.

1.5.4 Assessment of Plant Injury

At the conclusion of ethylene/ethane analysis, plants are removed from the bags and are maintained for three days using the growth conditions previously specified. At the end of the period, plants are reinspected for injury. Relative foliar injury, compared to negative controls, is expressed on a scale of 0 to 10 with each increasing unit being

*Cryovac Division, W.R. Grace and Co., Box 338, Simpsonville, SC 29681.

†The quantification of ethylene and ethane requires operation of the gas chromatograph at the limits of detection (2x background). The stated limit assumes optimum detector characteristics and may not be attainable on all instruments. A detection limit of 20 ppb may be used but cannot be exceeded. The detection limits should be included in the report. Alternate procedures may be obtained from the Process Measurements Branch, Air and Energy Engineering Research Laboratory, US EPA, Research Triangle Park, NC 27711.

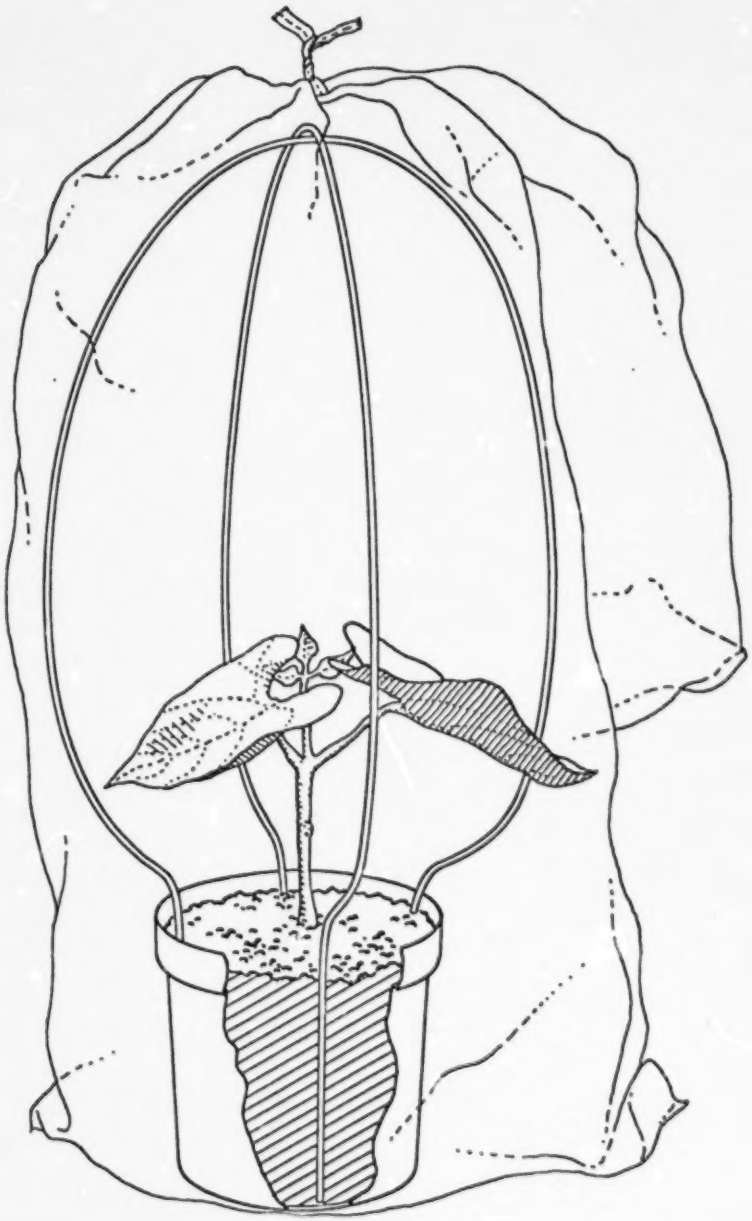

Figure 1.5 Plant Enclosure

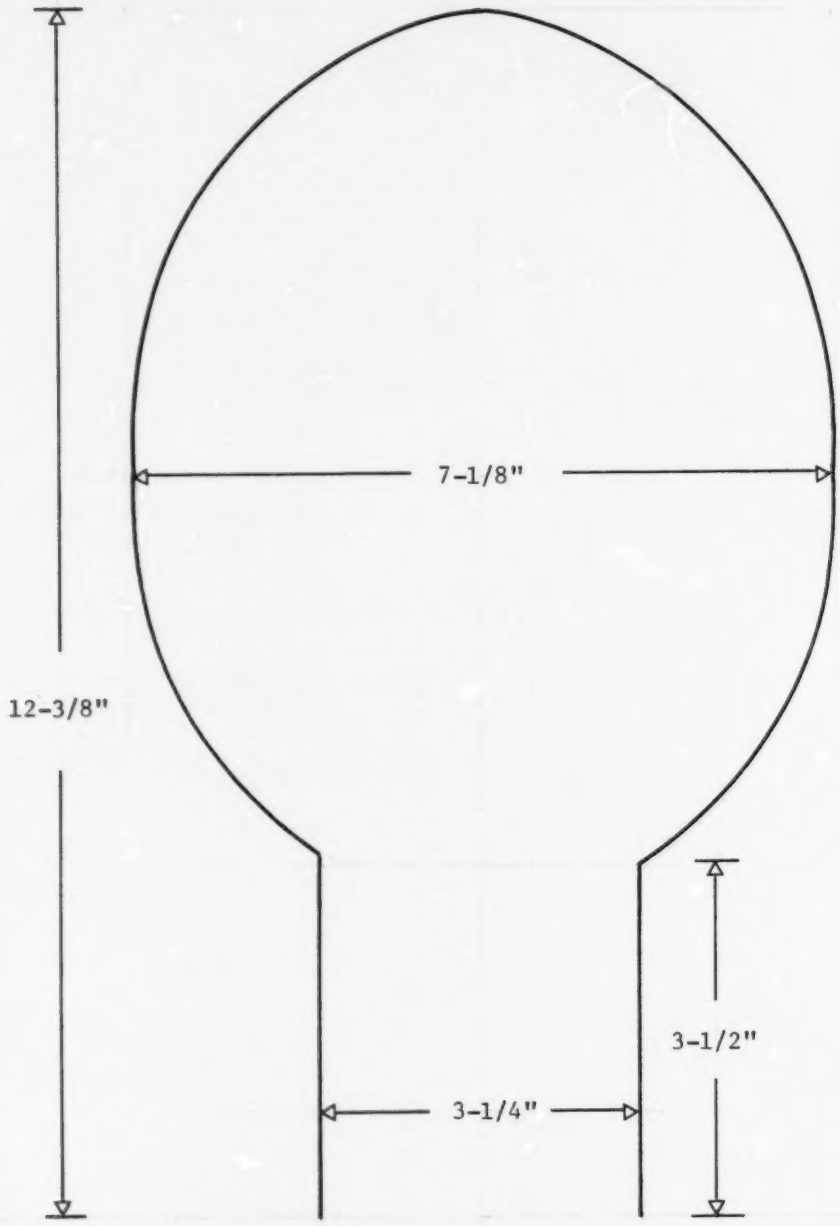

Figure 1.6 Encapsulation Frame

equal to 10 percent injury of the total leaf area. The types of injury (e.g. leaf lesions, yellowing) should also be noted. In the assessment of plant injury, any slight foliar injury must be reported as 1 (10 percent). Further, only the affected area is to be quantitated. For example, an entire leaf may be spotted, and the injury be assessed as 2 if only 20 percent of the leaf surface is necrotic or yellowed.

1.6 RESULTS AND DATA INTERPRETATION

Ethylene concentrations for plants of a given experimental condition are tabulated and the geometric mean and standard geometric deviation are determined. A stress response threshold value is determined which is 1.5 times the mean ethylene concentration of the negative control plants (to account for variability) or 50 parts-per-billion, whichever is greater. A positive response has occurred if the mean for any of the experimental groups exceeds this threshold. The response evaluated by the criteria in Table 1.1 is classed as "high" if the threshold is exceeded by the lowest test gas concentration (one minute); a "moderate" or "low" is given if the threshold is exceeded in the two-minute or seven-minute mixing times, respectively. Ethane data is monitored and recorded for future reference. Relative plant injury is reported as mean and standard deviation for a given experimental condition noting the type of injury which was observed.

TABLE 1.1 PLANT STRESS ETHYLENE TEST EVALUATION CRITERIA

Toxicity[a]	Lowest Positive Response (Mixing Time in Minutes)[b]	Relative Foliar Injury[c]
High	1	7-10
Moderate	2	4-6
Low	7	1-3
Not Detectable	No response at 7 minutes	0

[a]Use highest value parameter to rank to toxicity category.

[b]Dose levels based upon mixing time in minutes are applicable only if flow rates and chamber design given in this chapter are used.

[c]Evaluation based upon relative foliar injury are tentative and still under evaluation. Each unit is equal to 10 percent injury of total leaf area.

Due to the subjective nature of observation and assessment of plant injury, ranking of test samples based upon relative foliar injury is difficult and should not be used for definitive ranking of test material toxicity. Such information is useful to the investigator in the overall evaluation of a sample, and for recommendation for additional Level 1 or 2 testing. In the case of a highly toxic test gas which may damage the ethylene

production mechanism of the plant, visual foliar damage results are used to corroborate the highly toxic nature of the sample; little or no ethylene production with significant foliar damage (50 percent or greater at the one- or two-minute exposures) confirms a "high" toxicity evaluation. For evaluation of toxicity category the parameter showing the highest evidence of toxicity will be used.

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CHAPTER 2

LEVEL 1 ROOT ELONGATION BIOASSAY

by

Thomas A. Gezo
Union Carbide Corporation, Environmental Services
Tarrytown, NY 10591

SUMMARY

This section describes the Level 1 Root Elongation (RE) Test which is designed to assess the phytotoxicity of liquid effluent samples or aqueous extracts of particulate or solid samples. The bioassay employs germination and root elongation, two critical components of plant development, to assess the toxicity of effluents. Seed from five selected plant species are exposed to several concentrations of effluent and are incubated to allow early development. Inhibition of germination and root elongation is assessed with root elongation being more sensitive and the preferred endpoint.

2.1 INTRODUCTION

The assessment of phytotoxic effects requires the selection of a stage in plant development that is sensitive to a broad range of toxicants and is important physiologically. Seed germination and root elongation, critical links in plant development between a dormant embryo and a period of rapid growth with the formation of essential plant structures, were selected as end points for phytotoxicity testing. Toxic substances that prevent or reduce germination or root elongation will decrease plant populations and reduce crop yields. In natural systems affected species are less able to compete with other species and tolerant species may be selected, resulting in changes in species diversity, numbers and populations dynamics.

The inhibition of seed germination and root elongation has been used in determining selective toxicities of herbicides (1-3), screening plants for heavy metal (4-8) and salinity tolerance (9, 10), and evaluating toxic chemicals (11, 12) and allelopathic substances (13-16). The root elongation/seed germination bioassay is a rapid and simple test. Germination and root elongation can be observed after 115 hours of incubation and the test does not require large expenditures for equipment and facilities or complicated techniques.

A toxicant may cause responses at different doses in different plant species (12). To detect an effect from chemicals of unknown toxicity, several plant species are selected. The species used in this test are lettuce (Butter Crunch), Lactuca sativa L.; radish (Cherry Belle), Raphanus sativus L.; wheat (Stephens), Triticum aestivum L.; cucumber (Hybrid Spartan Valor), Cucumis sativus L.; and red clover (Kenland), Trifolium pratense L. These species are representative of economically important

plants and different plant families. The seeds chosen germinate and grow rapidly, contain no natural inhibitors, and require no special pretreatment. All test organisms are grown under identical environmental conditions (constant 25°C temperature, constant dark, and enclosed to maintain uniformly high relative humidity) and are exposed to several effluent concentrations.

Although inhibition of root elongation and germination are observable toxic responses, in this bioassay root elongation inhibition is the preferred end point. Usually, root elongation is inhibited at lower concentrations of toxic substances than is seed germination. Testing approaches are described, and criteria for interpretation are included.

2.2 TEST ORGANISMS

The seed used in the test are obtained from commercial seed companies, State Agricultural Experiment Stations and laboratories of U.S. Department of Agriculture. Seed from one seed lot for each species should be purchased in amounts adequate for one years' testing. Information on seed lot, the seed year or growing season collected and germination percentage should be provided by the source of seed. Only untreated (not treated with fungicides, repellants, etc.) seed is acceptable for Level 1 biological testing.

After purchase, seed are separated into size classes using a set of 4 hand screens (see Appendix 2.A), and by following the procedure in Appendix 2.B. The size class which contains the most seed is selected and used exclusively for the duration of the tests. When new seed is purchased the sizing is repeated for each species. After sizing, seeds are divided into small lots, placed in separate envelopes or sacks and stored in moisture-proof sealed containers (can or jar) in refrigerator at 5°C. Moisture content of the seed must be less than 10 percent.

2.3 TEST CHAMBERS

The test chambers used in the bioassay are constructed from glass tanks and have provisions for supporting five glass germination plates (one plate for each seed type). The glass tanks are 6-qt. (5.68 l) capacity and are constructed of one piece molded glass with approximate dimensions of 9-½ in. (24.13 cm) (L) x 6-¼ in. (15.88 cm) (W) x 7 in. (17.78 cm) (D) (Figure 2.1). (Manufacturers of glass tanks appear in Appendix 2.C.). Glass germination plates (5-1/8 x 6 in.; 13.02 x 15.24 cm) of single-strength window glass are prepared with polished edges. Germination plates are supported by a glass rack or by glass pegs. The use of glass racks has been found to be superior to the use of glass pegs. A single glass rack constructed from two 9 inch (22.86 cm) glass rods and six 4-3/4 inch (12.07 cm) (outside diameter) half circles of glass tubing connected to the rods at right angles at 1-3/8 in. (3.49 cm) intervals is used to support the glass plates at a 67° angle (Figure 2.2). Alternately, glass pegs (24 pegs/tank) that are 2-3 cm long and 5 mm in diameter (Figure 2.3) and cemented with epoxy (Duro-E-Pox-E hardner and resin, EPF-39 or equivalent) to the inside of the glass tank may be used to form a support for five glass plates at a 67° angle (Figure 2.4). Additional equipment and materials required for the bioassay appear in Appendix 2.D.

2.4 GENERAL EXPERIMENTAL PROCEDURES

2.4.1 Preparation of Glassware

The glass tanks fitted with glass racks or glass pegs, and glass plates are rinsed in acetone and then thoroughly washed in warm water with a synthetic detergent (Alconox® or equivalent). Following washing, the glassware is rinsed in tap water and in 50 percent nitric acid. All glassware is rinsed again thoroughly with tap water and distilled water.

2.4.2. Tissue Paper Pre-Cleaning

Eight to ten sheets of single-ply tissue (Kimwipes® or equivalent) are placed in a soxhlet extractor and extracted with distilled water for a minimum of 24 hours. After extraction, the tissues are removed, air dried, and stored in a dry container or plastic bag.

2.4.3. Test Medium

The test medium is an effluent sample or aqueous extraction of a particulate or solid sample. Samples should be tested as soon as possible after receipt to minimize sample alteration. If it is not possible to test immediately, the sample is stored at 0-4°C in a closed container. Leachates of solids are prepared using the Level 1 procedure below (17). Leachates of solid samples should be tested as soon as possible or the solid sample must be stored in closed polyethylene containers until extraction can be made. Dilutions of the effluent or aqueous extractions should be made without use of solvents or additives, except distilled water.

The highest concentration of test material that can be tested is one with an osmolarity of -3 bars so as to avoid osmotic effects which can retard root elongation and seed germination. Before testing, the pH of the samples and controls should be adjusted to 6.5 using KOH or HCL. The highest concentration that can be used is one for which the pH can be adjusted to 6.5 using less than 5 ml of 0.1M KOH or 0.1M HCl per liter of solution. No more than one percent organic solvent (usually dimethylsulfoxide) should be present in test sample.

2.4.4. Control Compounds

Negative controls should be run using distilled water as the test medium. For a positive control, it is recommended that cadmium chloride be used at a concentration of 15 mg/l for all seeds except wheat, for which 75 mg/l should be used.

2.4.5 Leachate Preparation

This procedure consists of shaking a known weight of solid with distilled water and separation of the aqueous phase by filtration.

The solid material will be sized and weighed. Large material should be ground to <3/8-inch (<9.5-cm) in diameter. The solid is placed in a 30-gallon (113.6-liter) linear-polyethylene drum. A volume of distilled water is added to the drum equal (in milliliters) to four times the weight (in grams) of sample. The container is then placed on a drum roller and agitated for 48 hours. The solution is allowed to settle; the supernatant decanted and pressure-filtered through a 0.45 µm membrane filter. The size of the container may be scaled down and bench-top shakers may be used when preparing small quantities of leachate.

2.4.6. Procedure for Planting Seed

Whatman 3MM chromatography filter paper rectangles (5-1/8 x 6 in.; 13.02 x 15.24 cm) or equivalent are soaked in the test solution in a shallow tray for a minimum of five minutes to saturate. One sheet of filter paper is removed from the test solution, allowed to drain and placed on a glass plate to which the paper adheres (Figure 2.5). Trapping air bubbles between the filter paper and the glass plate should be avoided. Using forceps, 15 seeds from one species are placed on the filter paper substrate in a row, equally spaced, across the top of the plate one inch (2.54 cm) down from the top edge (Figure 2.6). Seeds are placed with the radicle end toward the bottom of the plate and, in the case of wheat, with embryo side of the seed up (Figures 2.7-2.11). A narrow strip, (1-2 cm wide) of previously cleaned single-ply tissue is placed over the row of seeds to hold them in place and if necessary sprayed with just enough fine, distilled-water mist to cause the tissue to cling to the seed and filter paper (Figures 2.12, 2.13).

Five hundred to one thousand ml of test solution is poured into the rectangular glass tank fitted with glass peg guides (empty tank if glass rack is used). Normally, volumes less than 500 ml are not tested; however, samples with less than 500 ml can be tested if needed. Clean glass beads or marbles may be used to displace and raise the liquid level. The glass plate holding seed and substrate is inserted in the glass tank between the glass peg guides or in the glass rack, to support the plates at a 67° angle (Figure 2.14). The lower end of the plate opposite the seeds should be immersed in the test solution with a minimum of 2 cm, but not more than 3 cm, of the plate and filter paper in the solution. This procedure, with one plate of each seed type (cucumber, lettuce, radish, red clover, wheat) per container, is repeated with each test concentration and control.

2.4.7 Incubation

The glass tank, containing five plates with 15 seeds of each species per plate and the test solution, is enclosed in a heavy plastic bag and tied shut (Figure 2.15). The enclosed tank is placed in the dark, 25°C± 1.0°C controlled chamber. A tank is prepared for each test solution of sample effluent and for the controls.

2.4.8 Measurement of Root Length

Measurement of root length is made at 115 hours from the start of dark incubation. It is important to measure each plate as nearly as possible to 115 hours (not to exceed ± 30 min.). To measure root length, a plate is removed from the tank and placed on a flat surface. The lengths of all roots are measured to the nearest mm and entered on the data sheet. Measure from the transition point between hypocotyl and root to the tip of the root (Figure 2.16). At the transition between the hypocotyl and the primary root the axis may be slightly swollen, contain a slight crook, or change noticeably in size (radish, lettuce, cucumber, red clover). In wheat, the single longest primary or seminal root is measured from the point of attachment to the root tip. Transition points for each species are shown in Figures 2.17-2.21. There are additional descriptions and photographs helpful in making root measurements in the literature (18, 19).

2.5 TEST METHODS

Until the test has been conducted several times in a laboratory and is relatively routine, it might be best to conduct the range-finding test and definitive tests separately as described below. In the Level 1 manual (20), both tests have been combined into a single extended range-finding.

2.5.1 Range-Finding Test

The purposes of the range-finding test are 1) to determine if definitive testing with effluent is necessary, and 2) to aid in the selection of concentrations to be used in the definitive test when needed. The range-finding test consists of two negative control tanks, two tanks of 100 percent effluent and one tank each of 10, 1, 0.1 and 0.01 percent effluent.

A species need not be included in the definitive test if 1) both tanks containing 100 percent effluent had mean root lengths of at least 65 percent of control and 2) at least 10 of 15 seeds in one control and 8 of 15 seeds in the second control and 8 of 15 seeds in both 100 percent effluent tanks germinated. Also in this situation it is not necessary to examine the plates containing this species in the 10 to 0.01 percent tanks. If one or more of the species show mean root lengths less than 50 percent of the control at even the most dilute concentrations, it may be desirable to repeat the range-finding test using lower concentrations before proceeding to the definitive test.

2.5.2. Definitive Test

Estimation of an EC_{50} (concentration which reduces root elongation 50 percent) in this test will require two negative controls and at least six effluent concentrations chosen in a geometric series. A positive control is recommended. In a geometric series, the ratio of one concentration to the next is the same (e.g. 100, 50, 25, 12.5, 6.25 and 3.12 percent). If more than six concentrations are used, not all species must be tested at all concentrations. However, each species must be tested with at least six concentrations and those concentrations must be in a geometric

series. To accurately estimate the EC_{50} the following criteria must be met for each of the species except for any species which showed no effect with 100 percent effluent in the range-finding test. For the definitive test, both criteria 1 and 2 must be met and at least one criteria from among 3, 4 or 5 must be met.

2.5.3. Evaluation Criteria

1. At least 10 of 15 seeds on one control plate and 8 of 15 seeds on the other negative control plate must germinate.

And

2. Each effluent concentration in a series must be at least 50 percent as strong as the next concentration, except for the controls.

And at Least One of the Following

3. There must be at least one effluent concentration for which mean root length was above 65 percent of the control and one concentration for which it was below 35 percent of the control; both of these effluent concentration must have eight or more seeds germinate. In addition, any concentration less than the "above 65 percent" concentration must have a mean root length above 50 percent of the control and any concentration more than the "below 35 percent" concentration must have a mean root length below 50 percent of control.

Or

4. All conditions required in criteria 3 (preceding) must be satisfied with the sole exception that eight or more seeds need not germinate at the "below 35 percent" concentration. However, there must also be one concentration stronger than the "below 35 percent" concentration for which fewer than eight of fifteen seeds germinated.

Or

5. There must be two concentrations which each have mean root lengths of at least 65 percent of the control - at the lower concentration ten or more seeds must have germinated and at the higher concentration, five or fewer seeds must have germinated. In addition there must be one concentration higher than the "five or fewer" concentration for which seven or fewer seeds germinated.

Since most effluents affect root elongation at lower concentrations than germination, criterion 3 will usually be used to satisfy the requirements of the definitive test in addition to criteria 1 and 2. However, in cases where germination is inhibited at lower concentrations than elongation, it may be necessary to use criteria 4 or 5 in place of 3. If a species fails to satisfy criteria 1, 2 and either 3, 4, or 5, the definitive test must be repeated for that species.

2.6 CALCULATION OF AN EC_{50} FOR ROOT ELONGATION BY GRAPHICAL INTERPOLATION

Provided criteria 1, 2 and 3 are met in the definitive test, the EC_{50} can be estimated in the following manner. For each species which satisfied these 3 criteria, the effluent concentration on the logarithmic axis and the percent control mean root length on the arithmetic axis is plotted on semi-log paper. A straight line is drawn to connect at least three effluent concentrations used to satisfy criteria 3. Mean root length will be above 65 percent of control for one of these concentrations and the mean root length will be below 35 percent of the control for the other. The concentration at which this line crosses the 50 percent point for root length is the EC_{50} for root elongation. If no effects are seen with the 100 percent effluent or criteria 3 is not met due to germination inhibition (criteria 4 or 5 instead), it is not possible to estimate an EC_{50} for root elongation.

2.7 RESULTS AND DATA INTERPRETATION

For <u>each</u> of the species either the concentration in (a) <u>or</u> (b) <u>or</u> the quantities in (c) must be calculated and reported.

(a) If the species satisfied criteria 1, 2 and 3, an estimated EC_{50} for root elongation is reported. Graphical interpolation is used to estimate the EC_{50} and rank the test sample using evaluation criteria in Table 2.1.

TABLE 2.1 ROOT ELONGATION TEST EVALUATION CRITERIA[a]

Toxicity	Aqueous Liquids (EC_{50} in Percent Effluent)
High	<0.01
Moderate	0.01-1
Low	1-100
Not Detectable	>100

[a]Criteria for aqueous liquids are tentative and still under evaluation. Criteria for other sample forms (such as soluble solid and aqueous leachates of insoluble solids) are under development.

(b) If the species satisfied criteria 1 and 2 but not criteria 3 (criteria 4 or 5 are used instead), the lowest concentration for which fewer than 8 of 15 seeds germinated is reported. The EC_{50} cannot be estimated for root elongation or inhibition of seed germination from data in this category. Currently, test samples are not ranked from data of this type.

(c) If the range-finding test showed that 100 percent effluent had no effect on a species, the number of seeds which germinated and percent control mean root length for the two 100 percent effluent tanks is reported. The test sample is ranked as having nondetectable (ND) toxicity.

In addition, data sheets should be completed during the course of the bioassay. The data forms and instructions for use of the data forms are included in Appendix 2.E. When the bioassay on an effluent is completed, these sheets must be returned for both the range-finding and definitive tests.

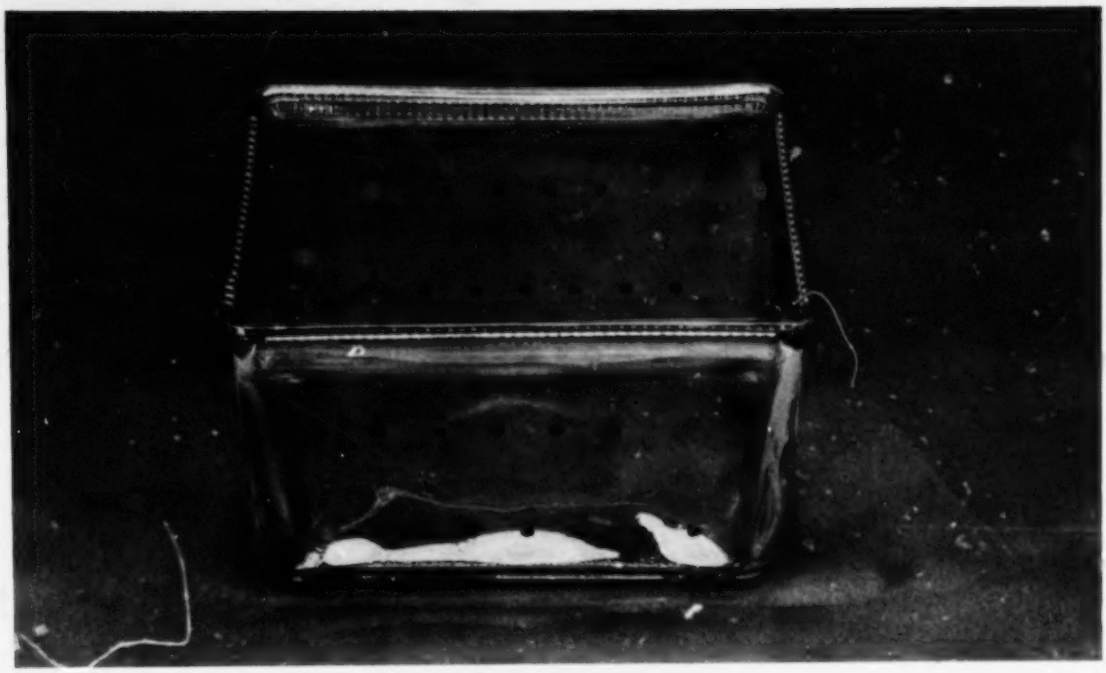

Figure 2.1 Glass Tank

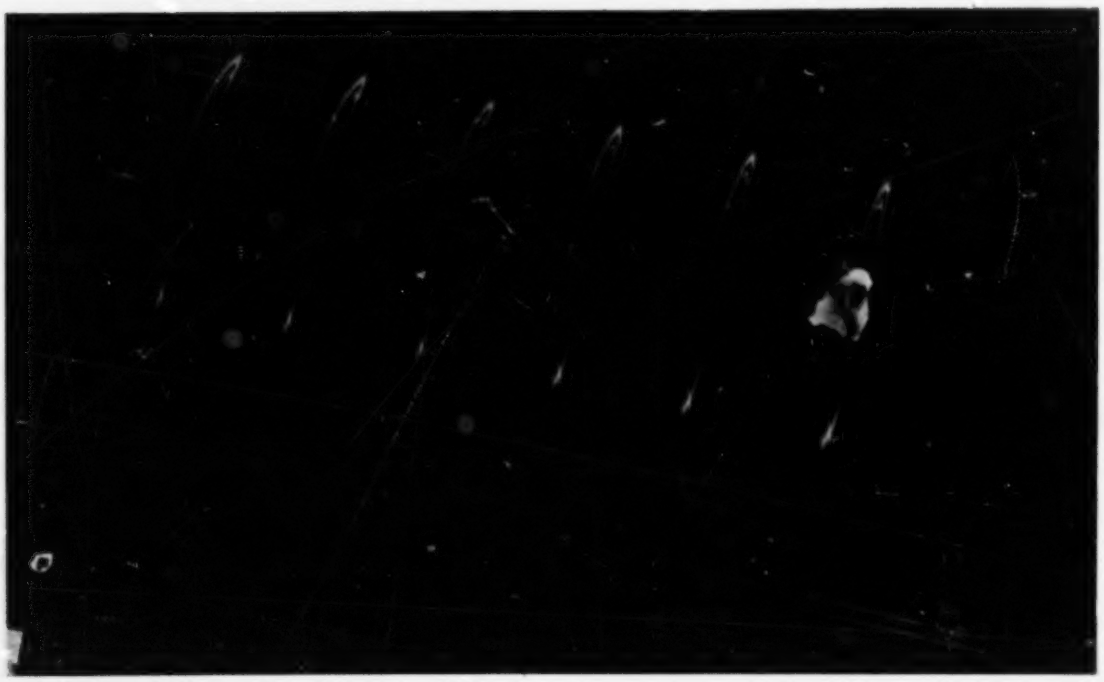

Figure 2.2 Glass Rack

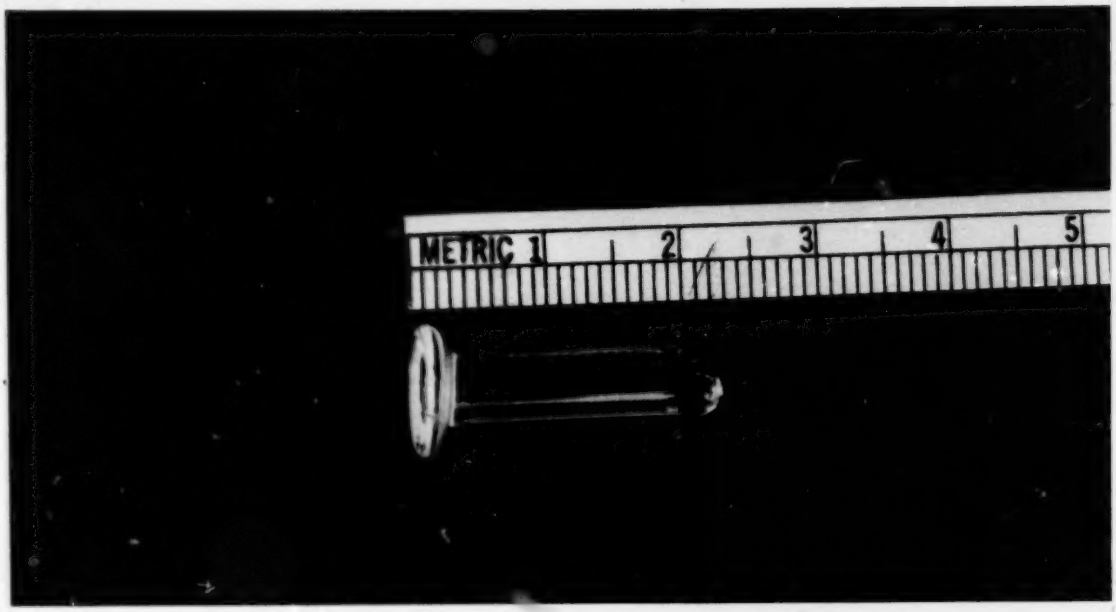

Figure 2.3 Glass Peg, Showing Size and Shape

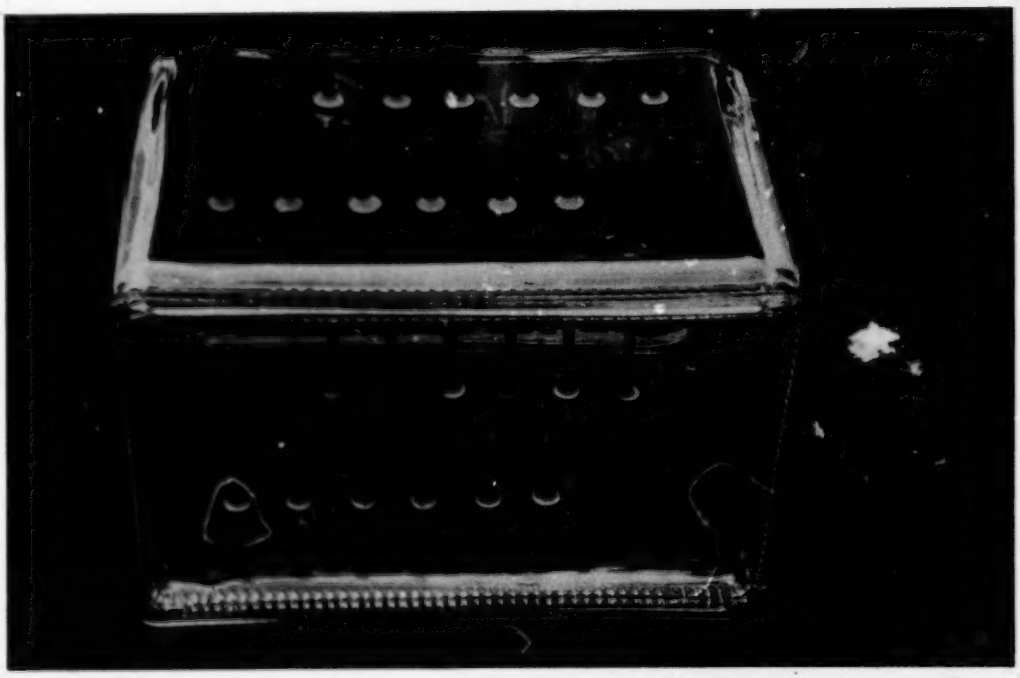

Figure 2.4 Glass Tank With Glass Pegs Cemented in Place

Figure 2.5 Filter Paper Being Positioned on Glass Plate

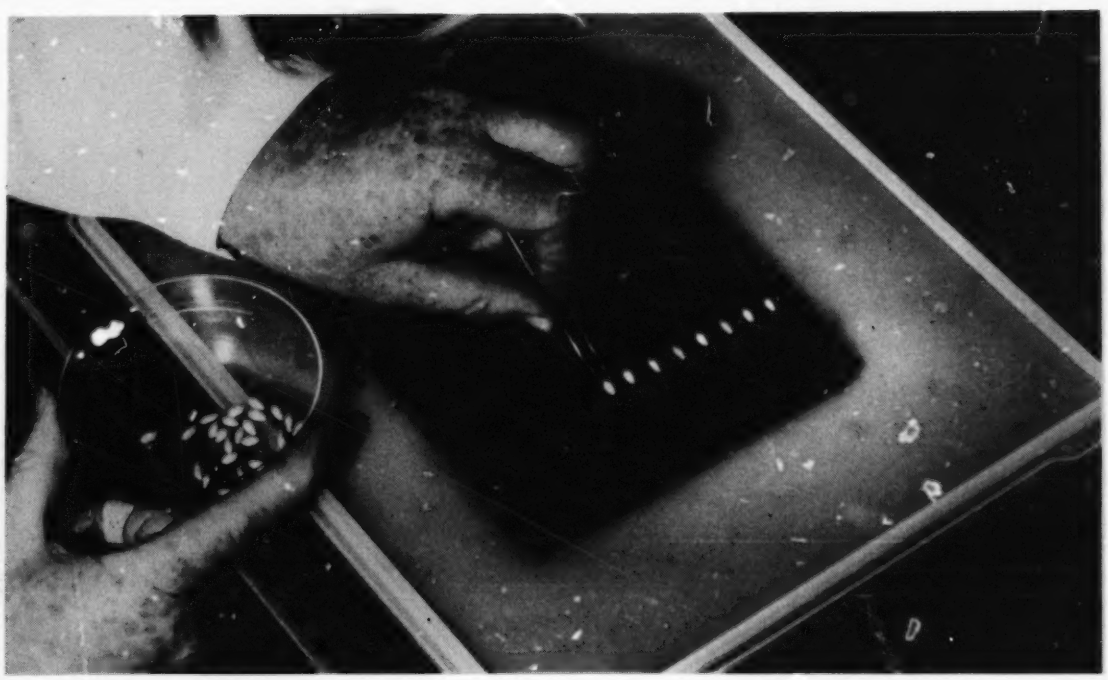

Figure 2.6 Planting Seed on Filter Paper Substrate

Figure 2.7 Wheat Seed With Embryo Side Up and Radicle End Toward the Bottom

Figure 2.8 Cucumber Seed With Radicle End Toward the Bottom

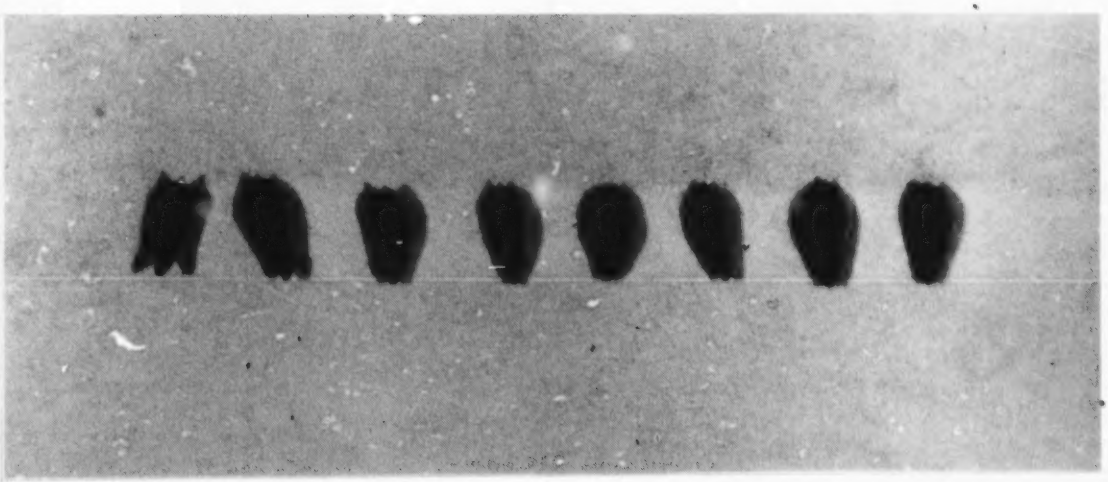

Figure 2.9 Lettuce Seed With Radicle End Toward the Bottom

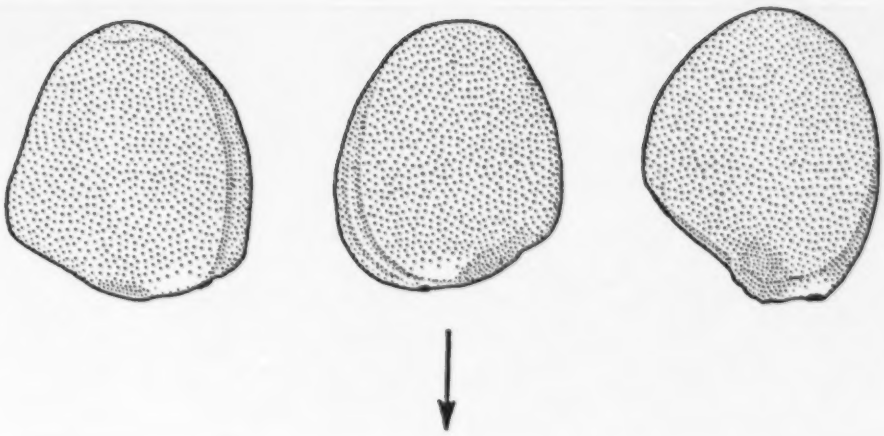

Figure 2.10 Radish Seed With Radicle End Toward the Bottom

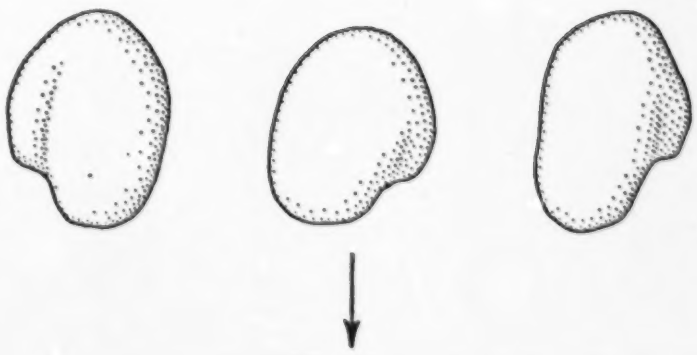

Figure 2.11 Red Clover Seed With Radicle End Toward the Bottom

Figure 2.12 Tissue Strips Covering Row of Seed

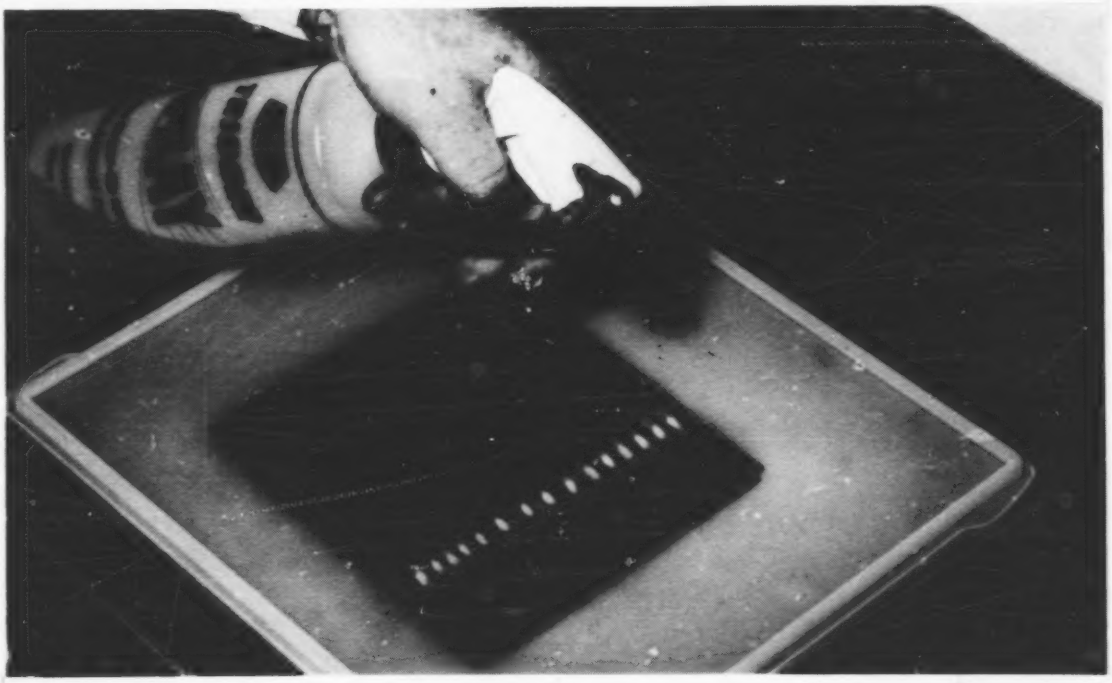

Figure 2.13 Spray Bottle With Mist Being Applied to Seed and Tissue Strip

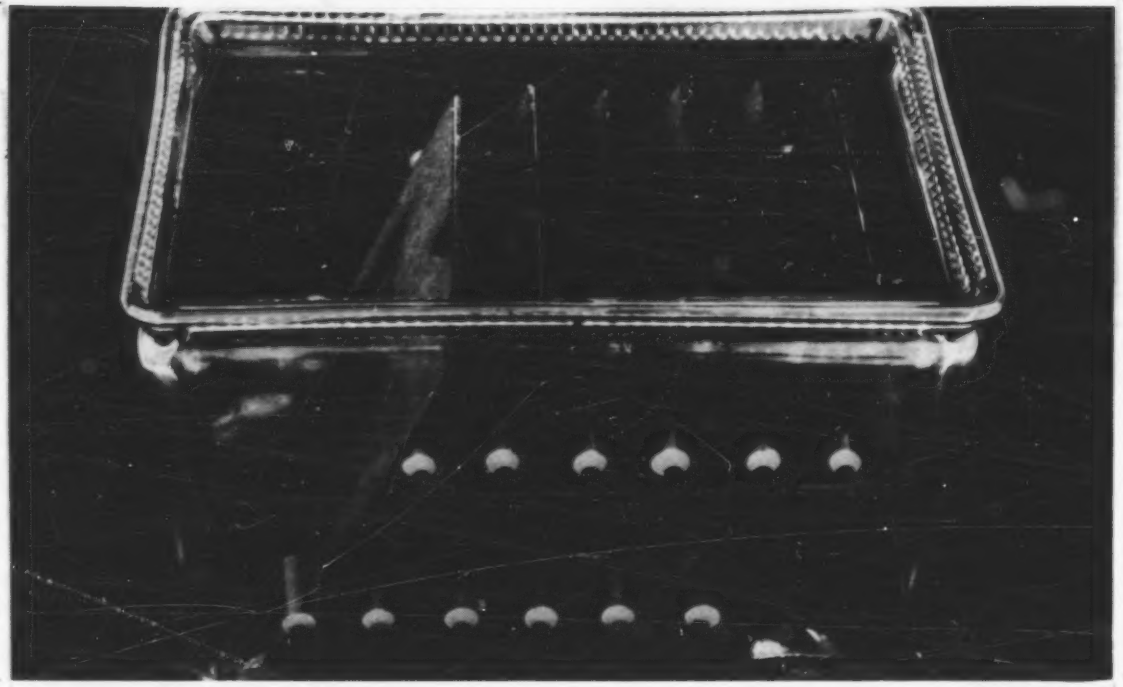

Figure 2.14 Glass Tank With Glass Plates in Position Between Pegs

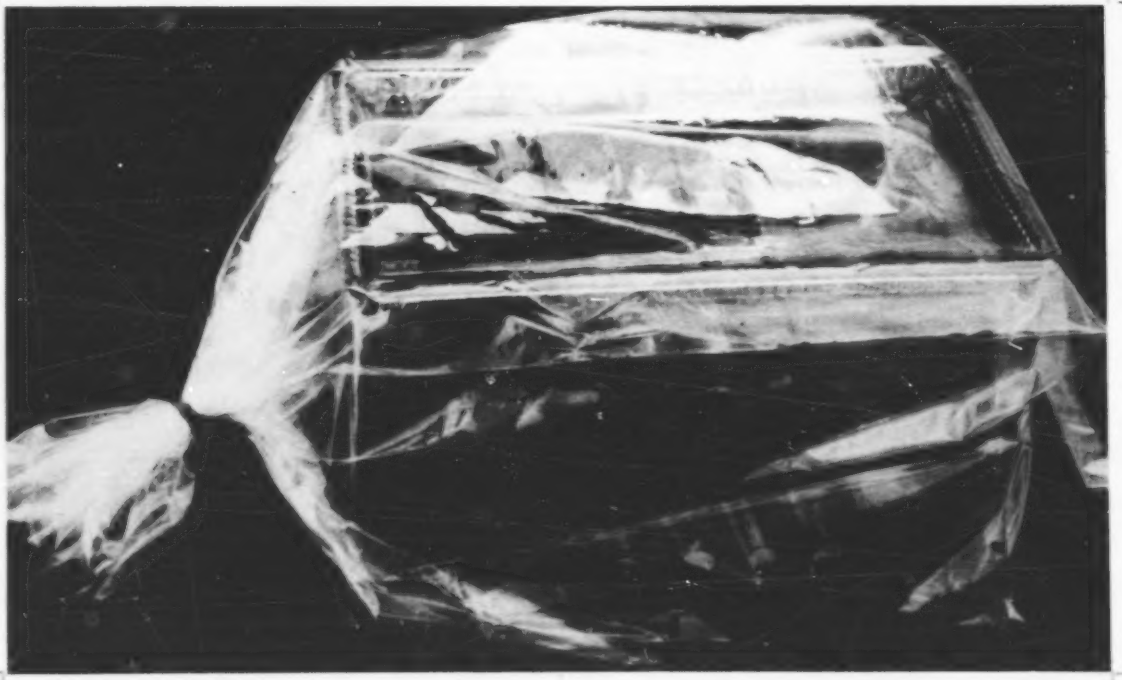

Figure 2.15 Chamber With Plates Covered For Incubation

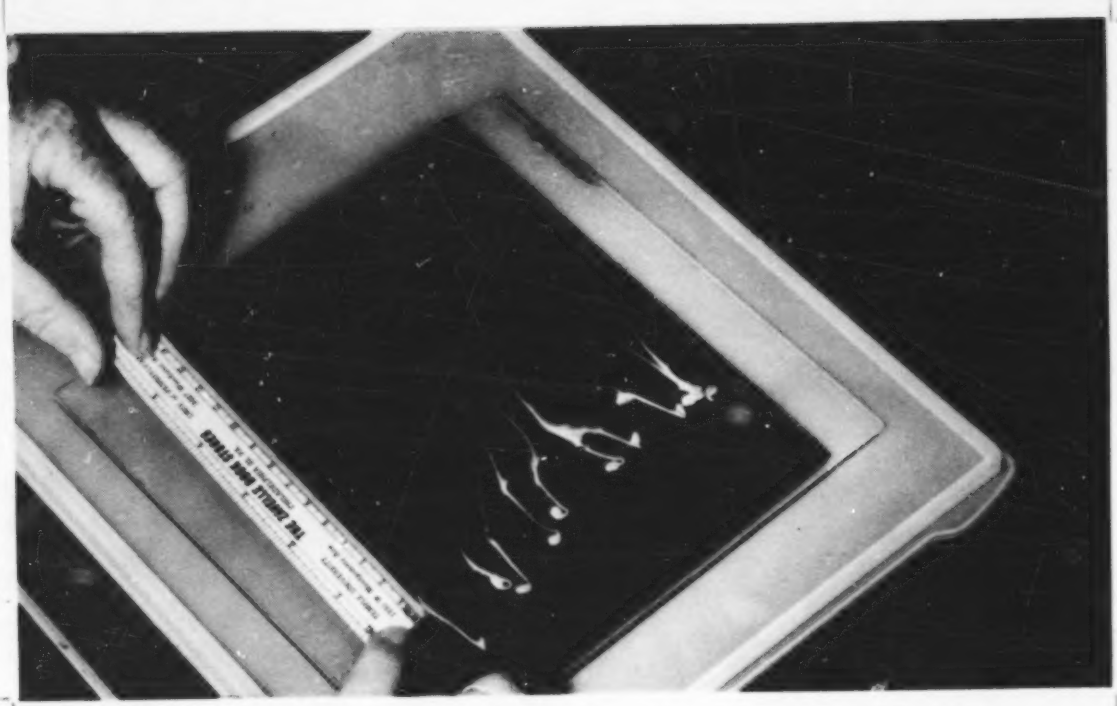

Figure 2.16 Seedlings Showing Method of Measuring Roots

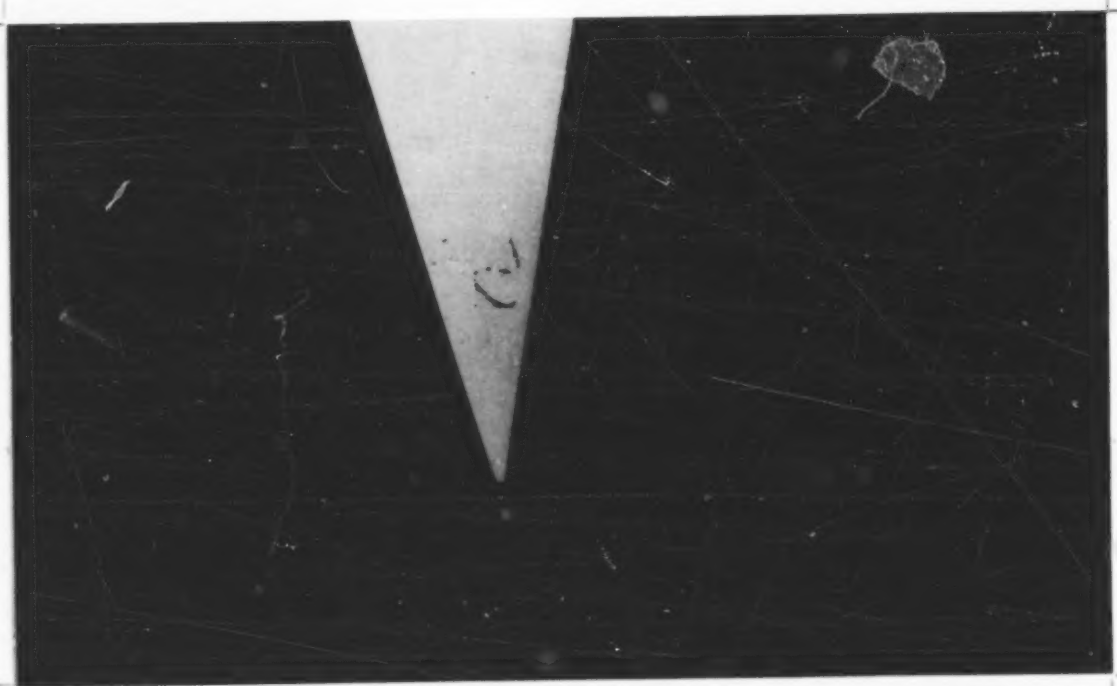

Figure 2.17 Lettuce Seedling With Pointer Showing Transition Point Between Root and Hypocotyl

Figure 2.18 Radish Seedling With Pointer Showing Transition Point Between Root and Hypocotyl

Figure 2.19 Cucumber Seedling With Pointer Showing Transition Point Between Root and Hypocotyl

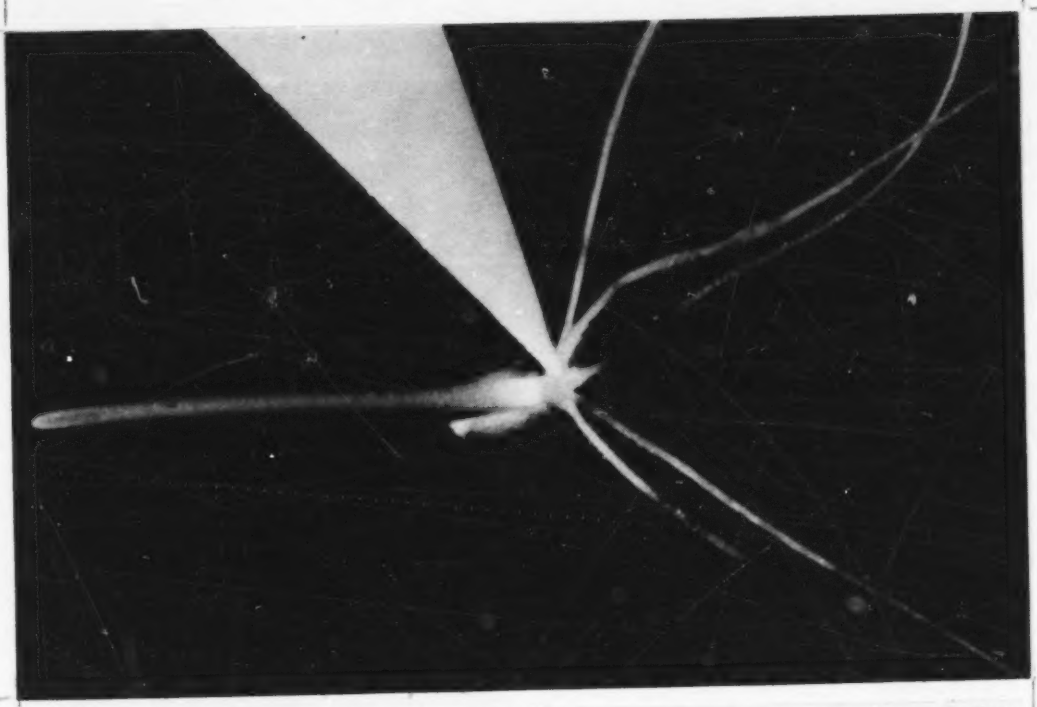

Figure 2.20 Wheat Seedling With Pointer Showing Transition Point Between Root and Hypocotyl

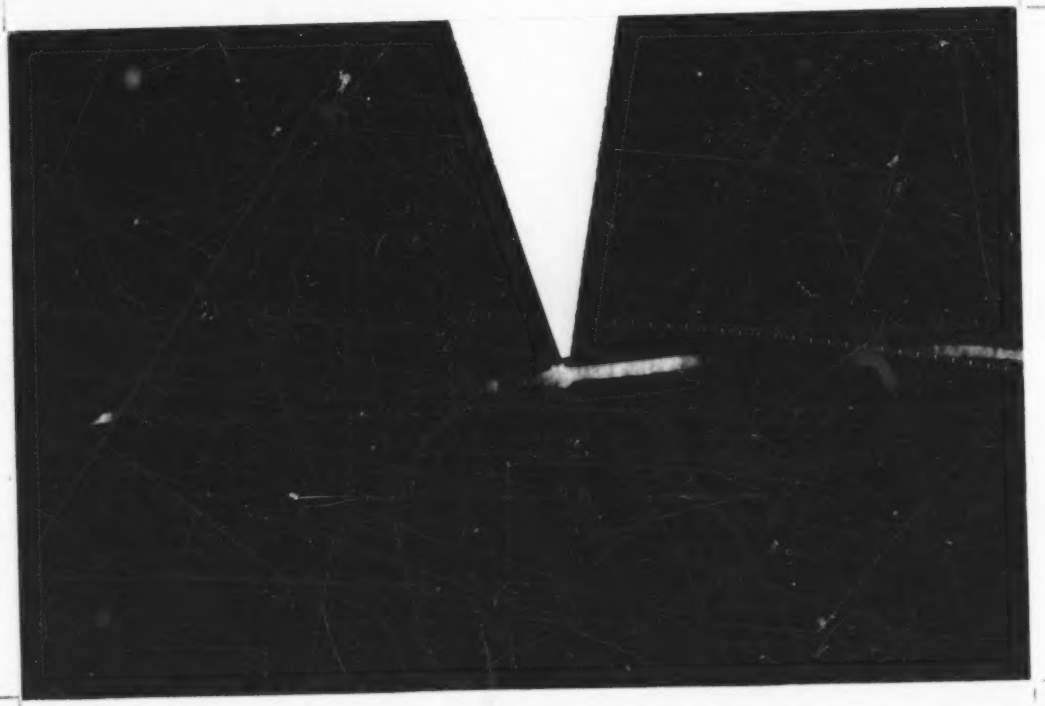

Figure 2.21 Red Clover Seedling With Pointer Showing Transition Point Between Root and Hypocotyl

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APPENDIX 2.A

HAND SCREENS (SEED DOCKAGE SIEVES)

Sizes of Perforations and Wire Mesh for Screens to Use in Sizing Seed

Species	Perforated Metal Sheet		Wire Mesh
	Round Holes	Oblong Holes	
Red Clover	1/19, 1/18, 1/17, 1/16 (fractions of an inch)		
Radish	6 1/2, 7, 7 1/2, 8 (64ths of an inch)		
Wheat	9, 9 1/2, 10, 10 1/2 (64ths of an inch)		
Cucumber		1/13 x 1/2 1/14 x 1/2 1/15 x 1/2 1/16 x 1/2 (fractions of an inch)	
Lettuce			6 x 28 6 x 30 6 x 32 6 x 34 (fractions of an inch) 1/16" x 1/28"

Manufacturers: A.T. Ferrell and Co.
Saginaw, MI 48601
(517) 792-8723

Seedburo Equipment Co.
1022 W. Jackson Blvd.
Chicago, IL 50607
(312) 738-3700

APPENDIX 2.B

PROCEDURE FOR SIZING SEED USING HAND SCREENS

The size grading is carried out on the entire seed lot for each kind of seed. Small samples of 100-150 g are sized at a time.

1. Remove trash, empty hulls, and damaged seed from the entire seed lot.

2. Depending on species, select a series of four screens to separate sample into size classes (see Appendix 2.A).

3. The four screens are nested with the screen containing the largest holes on top and screens with successively smaller holes in sequence below. A blank or bottom pan collects that fraction which passes through all the screens.

4. Seed is poured on the top screen and the whole set of nested screens are shaken by hand or with a vibrator until all the seed remain on one of the screens or reach the bottom pan.

5. The separated fractions are collected and the procedure is repeated until all the seed in the lot are sized.

6. The fractions are labeled as to size and prepared for storage.

APPENDIX 2.C

SUPPLIERS OF GLASS TANKS

Manufacturer

Anchor-Hocking Glass Co.
70 Glass Avenue
Lancaster, OH 43130
(614) 687-2111

Distributor

Carolina Biological Supply Co.
Burlington, NC 27215
(919) 584-0381
Cat. No. 67-0460 All-glass rectangular aquarium

Merchants Paper Co.
1206 SE Union
Portland, OR 87214
Stock No. 91 glass tank

APPENDIX 2.D

LABORATORY EQUIPMENT AND MATERIALS

D.1 APPARATUS

Glass Test Chambers	one piece molded glass tank, 6 quart (5.68 liter) capacity approximately 2-½ in. (24.13 cm) (L) x 6-¼ in. (15.88 cm) (W) x 7 in. (17.78 cm) (D) with glass pegs or rack (Appendix 2.C for manufacturers).
Glass plates	5-1/8 x 6 inches (13.02 x 15.24 cm) single strength window glass with polished edges.
Misc. glassware	pipets, graduated cylinders, beakers and other glassware associated with a chemical or research laboratory.
Filter paper	Whatman 3MM chromatography paper, 46 x 57 cm sheets.
Single ply Kimwipe Tissue	
Spray bottle	fog nozzle or similar watering head to emit fine spray or mist.
Metric measuring device	metric scale or template.
Forceps	watchmakers forceps or other fine type to orient seeds on the filter paper substrate.
Illuminated magnifier	may be helpful for planting and for seedling examination and root measurements.
Sample/storage bottles	glass, polyethylene or polypropylene.
Plastic bags	minimum size of 12 x 8 x 14 inches (30.5 x 20.3 x 35.6 cm) to enclose glass tanks.
Soxhlet extraction apparatus	
pH Meter	scale range 0-14 pH units, ± 0.1 pH.
Balance	200 to 300 g capacity, ± 0.1 g precision
Tray or shallow pan	glass or polyethylene shallow receptacle with sides of approximatley 1-½ to 2 inches (3.8 to 5.1 cm).
Hand Screens	9 x 9 inches (22.9 x 22.9 cm) (for required sizes and manufacturer see Appendix 2.A)

D.2 REAGENTS

Nitric acid (50 percent)
Acetone
Alconox®
Distilled Water

D.3 EQUIPMENT AND FACILITIES

The facilities must include work areas for planting seed and for measurements, preferably isolated from other activities. There should be a fume hood that may be used in cleaning glassware and in planting and measuring when testing potentially hazardous substances. Distilled water is necessary for cleaning glassware and dilution of sample effluent. Refrigeration at 5°C is required for supplies and seed storage. The test facility must have a seed germinator, plant growth chamber or some type of controlled environment chamber capable of maintaining a uniform temperature at 25°C with ± 1.0°C.

APPENDIX 2.E

INSTRUCTIONS FOR DATA FORMS

1. General -- There are two types of data forms; a raw data form labeled with 1 in the upper left hand corner (Table 2.E.1), and a summary form labeled with 2 (Table 2.E.2 and continued on Table 2.E.3). Record the data for individual seeds on the raw data form (#1) Number of seeds germinated and mean root length are also entered on this form. On the summary form (#2) record number of seeds germinated, mean root length and percent control mean root length for each plate. Fill out one #1 form for each tank and one #2 form for each test. Sample forms follow the instructions.

2. General Data Entry -- Except for concentration, do not use any decimal points. Round Percentages to the nearest whole percent and root lengths to the nearest mm. Except for concentration, enter numbers as far right as possible - any blank boxes should be on the left of a data entry.

3. Test Number -- Always enter a 1 for the range-finding test and 2 for the definitive test. For any additional definitive test use 3, 4, etc. In the case of a second range-finding test, enter a -2.)

4. Missing Data -- Simply leave a space blank when data on a seed is unavailable. Missing data should be noted in the comments section of the #2 form. Indicate concentration, species, and number of seeds missing.

5. Date -- Enter the date as month-day-year using the first 2 boxes for month, the next 2 for the day and the last 2 for the year.

6. Concentration -- Enter as percent effluent. Note that the concentration for the negative controls, "0.0", is already entered on lines 1 and 2 of Form 2. Positive controls, if used, should be entered and identified using Form 1, and summarized under the appropriate tank number on Form 2.

7. Germination Code -- Enter the germination code in the columns labeled "G" on the raw data form (#1). Germination code:
   - 0 - No emergence of radicle or emergence of plant structures with insufficient development for measurement of root length.
   - 1 - Emergence of essential plant structures with normal or abnormal development.

8. Number Germinated -- Enter the number of seeds which germinated on a plate. This will equal the number of seeds on the plate which had a germination code of 1.

9. Mean Root Length -- Compute the mean using only seeds with a germination code of 1.

10. Percent Control -- Compute the average of the mean lengths of the two negative control plates and use this average in determining percent control.

11. Comments -- If the results of a test are invalid, use the comments section to indicate what happened. Any other problems or unusual circumstances can be noted here using the back of the sheet if necessary.

TABLE 2.E.1 RAW DATA FORM (ROOT ELONGATION TEST)

1 1

LAB # 2 3

EFFLUENT CODE 5 6 7

EFFLUENT NAME _____

TEST # 9 10

STARTING DATE _____ ENDING DATE 12 13 14 15 16 17

CONCENTRATION % 19 20 21 22 23 24 25 26 27 28 29 30 31 32

	33 34 35	G 37	LENGTH 42 43 44	G 46	LENGTH 51 52 53	G 55	LENGTH 60 61 62	G 64	LENGTH 69 70 71	G 73	LENGTH 78 79 80
RED CLOVER	1 6 6										
		# GERM 42 43 44			MEAN LENGTH 51 52 53						
LETTUCE	0 5 6										
WHEAT	1 6 5										
CUCUMBER	1 6 3										
RADISH	0 1 1										

TABLE 2.E.2 SUMMARY FORM (ROOT ELONGATION TEST)

1 2

LAB # 2 3

TEST # 9 10

EFFLUENT CODE 5 6 7

EFFLUENT NAME _____

STARTING DATE _____

ENDING DATE 11 12 13 14 15 16

Did Anything Happen During TEST to Invalidate Results? (Enter 0 for No, 1 for Yes) 18
If Yes, Comment Below.

WAS CRITERIA 1,2 AND EITHER 3,
4 OR 5 SATISFIED
(0 FOR NO, 1 FOR YES)

TANK 20 21

CONCENTRATION % 22 23 24 25 26 27 28 29 30 31 32 33 34 35

1 — 0.0

2 — 0.0

MEAN LENGTH OF 2 CONTROLS

TANK 20 21

CONCENTRATION % 22 23 24 25 26 27 28 29 30 31 32 33 34 35

3, 4, 5, 6, 7, 8, 9, 10, 11, 12

RED CLOVER 39			LETTUCE 48			WHEAT 57			CUCUMBER 66			RADISH 75		
# GERM 36 37 38	MEAN LENGTH 39 40 41		# GERM 45 46 47	MEAN LENGTH 48 49 50		# GERM 54 55 56	MEAN LENGTH 57 58 59		# GERM 63 64 65	MEAN LENGTH 66 67 68		# GERM 72 73 74	MEAN LENGTH 75 76 77	
# GERM 36 37 38	MEAN LENGTH 39 40 41	% CONTROL 42 43 44	# GERM 45 46 47	MEAN LENGTH 48 49 50	% CONTROL 51 52 53	# GERM 54 55 56	MEAN LENGTH 57 58 59	% CONTROL 60 61 62	# GERM 63 64 65	MEAN LENGTH 66 67 68	% CONTROL 69 70 71	# GERM 72 73 74	MEAN LENGTH 75 76 77	% CONTROL 78 79 80

COMMENTS:

TABLE 2.E.3 CONTINUATION SUMMARY FORM (ROOT ELONGATION TEST)

1 2

LAB # 2 3

TEST # 9 10

EFFLUENT CODE 5 6 7

STARTING DATE _____

EFFLUENT NAME _____

ENDING DATE 11 12 13 14 15 16

Did Anything Happen During TEST to Invalidate Results? (Enter 0 for No, 1 for Yes) 18
If Yes, Comment Below.

MEAN LENGTH OF 2 CONTROLS

TANK 20 21	CONCENTRATION % 22 23 24 25 26 27 28 29 30 31 32 33 34 35	RED CLOVER 30 # GERM 36 37 38	RED CLOVER MEAN LENGTH 39 40 41	RED CLOVER % CONTROL 42 43 44	LETTUCE 48 # GERM 45 46 47	LETTUCE MEAN LENGTH 48 49 50	LETTUCE % CONTROL 51 52 53	WHEAT 57 # GERM 54 55 56	WHEAT MEAN LENGTH 57 58 59	WHEAT % CONTROL 60 61 62	CUCUMBER 66 # GERM 63 64 65	CUCUMBER MEAN LENGTH 66 67 68	CUCUMBER % CONTROL 69 70 71	RADISH 75 # GERM 72 73 74	RADISH MEAN LENGTH 75 76 77	RADISH % CONTROL 78 79 80
1 3																
1 4																
1 5																
1 6																
1 7																
1 8																
1 9																
2 0																
2 1																
2 2																

COMMENTS:

CHAPTER 3

LEVEL 1 INSECT TOXICITY BIOASSAY

by

David J. Brusick
5516 Nicholson Lane
Kensington, MD 20895

SUMMARY

This chapter describes the Level Insect Toxicity (IT) Bioassay which is designed to assess and rank the acute toxicity, and additionally, the reproductive toxicity of liquid, soluble solid or fine particulate effluents. Study designs include range-finding toxicity test, definitive toxicity test for LD_{50} determination and an optional fertility test. This section describes the culture of test organisms (Drosophila melanogaster), preparation of test materials for dosing, study design procedures, interpretation of results, and ranking test material toxicity.

3.1 INTRODUCTION

Drosophila melanogaster is a common insect species in nature; many features of this organism make it attractive as an in vivo test system for detecting environmental toxicants. These features include: 1) a short life cycle of 10-12 days; 2) minimal space, monetary, and manpower requirements to maintain stocks; 3) the ability to detect toxic effects at specific life cycle stages (adult, germinal, and developmental); 4) a well-defined genetic system which makes the detection of specific genetic end points possible; 5) the ability to biotransform genotoxic chemicals via in vivo metabolic enzyme systems; and 6) amenability to exposure to liquids, soluble solids, and small particles. Feeding via solution is the most common route of exposure.

Drosophila is included in EPA Level 1 environmental assessment testing because of its application as a toxicity screening test and also because it can be expanded into testing for additional end points. For Level 1 applications, Drosophila will be used to assess acute toxicity in the adult flies and reproductive capacity among surviving flies following exposure to, or treatment with, environmental samples. The acute toxicity data will be used to rank effluent streams and identify sources for further testing. The fertility response will be recorded and may be used to provide possible direction for testing at Level 2.

3.2 MATERIALS AND METHODS

3.2.1 Stocks

Drosophila melanogaster can be easily found in nature but pedigreed stocks may be obtained from the Drosophila Stock Center*. One-day-old, wild type Oregon R (Ore R) male flies are used for exposure to or treatment with environmental samples. One-day-old Ore R virgin female flies are used for mating and egg production in fertility tests. The life cycle steps of this organism covers approximately 10 days when the flies are grown at 25°C and 60 percent relative humidity. The steps from adult to adult are described below.

TABLE 3.1. DROSOPHILA LIFE CYCLE

Life Cycle Stage	Duration
Embryonic Development in Eggs	1 day
First Larval Instar	1 day
Second Larval Instar	1 day
Third Larval Instar	2 days
Prepupa	4 hours
Pupa	4.5 days

Male flies become sexually mature within 6-12 hours after hatching from pupae (Figure 3.1). Females collected during this period from a cleared vial will therefore be virgins (Figure 3.1).

3.2.2 Culture Media

Several types of culture media may be used to maintain stocks of Drosophila. Formulation of a typical cooked medium is given below. Instant Drosophila medium† (Formula 4-24 without dyes), can also be used in preparing culture bottles and vials. The flies are maintained on the medium in 8 dram glass vials plugged with nonabsorbant cotton. Stock cultures should be placed on new medium every two or three weeks. The generation time of Drosophila can be extended to almost four weeks by maintaining the stocks at 18°C. This technique is useful in holding

*Drosophila Stock Center, Bowling Green, KY 42101.

†Carolina Biological Supply Company, Burlington, NC 27215.

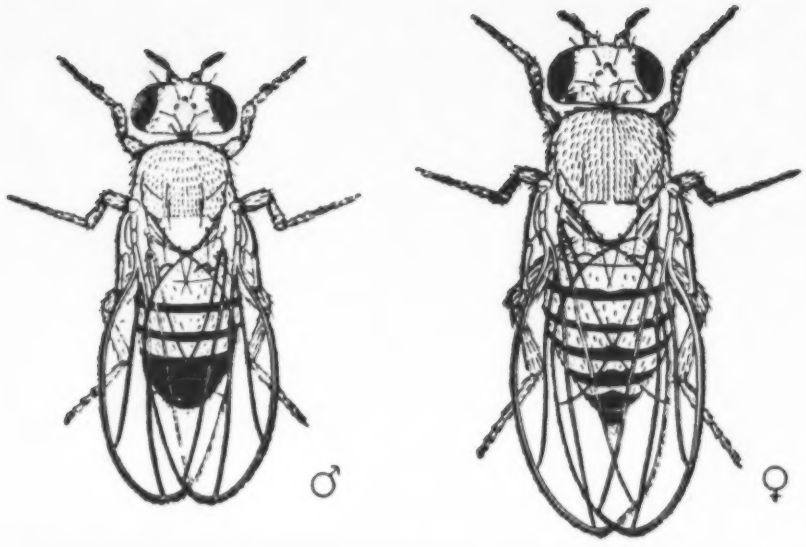


Figure 3.1 The Morphology of male and female *Drosophila* [1]

TABLE 3.2. DROSOPHILA CULTURE MEDIUM

Ingredient	Amount
Water	1.3 liter
Yellow Corn Meal	710 g
Brewer's Yeast	710 g
Agar	110 g
Methyl-p-hydroxybenzoate (Methyl Parasept)	38 g
Propionic Acid	80 ml

stocks between experiments. The biology and handling of Drosophila melanogaster are described in more detail in References 1 and 2.

The powder components are added to the water, lumps broken up and the mixture is brought to a boil and cooked for 15 minutes. Propionic acid is then stirred into the molten medium and it is dispensed into culture vials (8-12 ml/vial). Once dispensed, the vials are placed in racks and covered with sterile gauze until completely set. The medium-containing vials are then plugged with nonabsorbent cotton and stored at 4°C until used. A small amount of Baker's yeast may be added to the surface to facilitate egg laying.

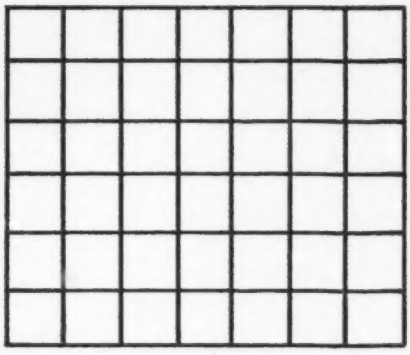
The handling of flies is rather easy after a short practice period. Adult flies can be transferred rapidly from vial to vial by gently tapping the vial against the palm of the hand to cause flies to fall to the bottom followed by shaking them into the new vial and replugging. Prolonged observation and manipulation requires anesthetization of the flies with either ether or CO_2. A simple etherizer has been described by Demerec and Kaufman (1) and a device for CO_2 anesthetizing flies has been described by Würgler et al. (3).

3.3. EXPERIMENTAL DESIGN

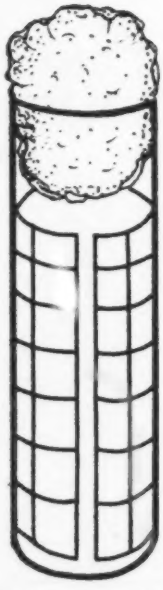
3.3.1 Feeding Study

Drosophila may ingest particles suspended in liquid but are generally exposed to liquid or solid samples in solution. Liquid environmental samples are tested with various solvents such as water, DMSO, and ethanol to determine the most suitable solvent or feeding medium. Water soluble samples are dissolved directly in a 1 percent sucrose feeding solution. Liquid samples that are not soluble in water are solubilized in ethanol or DMSO and then added to a sucrose feeding solution. The feeding solution is applied evenly to filter paper at appropriate concentrations. The filter papers are allowed to dry and are then placed in a dosing vial as shown in Figure 3.2. Liquid samples not soluble in water, ethanol,

Glass Culture Vial
Plugged With Cotton Plug



Filter Paper Liner[Solubilized compound is added , spread evenly, and dried.]



Vial Prepared For Exposure. [Fifty flies are added to the vial for 24 hours.]

Figure 3.2 Preparation of Treatment Chambers for *Drosophila*

or DMSO are mixed into a thick paste made with yeast extract and 1 percent sucrose water solution to form an emulsion mixture which is fed to the flies by applying it to the filter paper liner.

Solid samples are dissolved in 1 percent sucrose feeding solution if soluble. Otherwise they are processed into fine particles not larger than 5 μm and mixed with 1 percent sucrose solution to form a suspension. Both solutions and suspensions are applied to filter paper liners for feeding. Exact dose levels (total consumed sample) are not easily determined for Drosophila.

The flies are maintained in dosing vials for 24 hours. The number of flies surviving treatment are counted at the end of the exposure period and the living flies are transferred to new vials with medium to measure reproductive capacity (Fertility Test). Methods for gaseous and injection exposures are presently in development.

3.3.2 Range-Finding Test

For feeding experiments, groups of 50 male flies which have been starved for two hours, are placed in vials containing 1.5 ml of feeding solution on filter paper at test agent concentration levels of 0, 0.1, 1, 10 and 50 mg/ml for solids and 0, 1, 10, 100 and 500 μl/ml for liquids, or containing 5 g of yeast paste with test agent concentration levels of 0, 0.1, 1, 10 and 50 mg/g or 0, 1, 10, 100 and 500 μl/g. The vials are scored for dead flies after 24 hours and a range of concentrations for LD_{50} evaluation is developed including levels of the test sample which produce some lethality.

3.3.3 Definitive Test for LD_{50} Determination

For feeding studies, the LD_{50} determination is performed by using five equally spaced concentration levels derived from the information obtained from the range finding test. A total of 150 starved male flies, 50 per culture vial, are fed the test sample at each specified concentration level for 24 hours.

Dead flies are counted immediately at the end of the feeding or exposure period and the 24-hour LD_{50} and its upper and lower limits are calculated according to Litchfield and Wilcoxon (4).

3.3.4 Optional Fertility Test

Twenty-five to fifty male flies obtained from each dose level of the toxicity tests are individually mated to three virgin females in food vials. After four days, the adults are discarded. The vials are examined daily for five to eight days after exposure for evidence of larval activity. The absence of larvae or a lack of normal larval activity as compared with control flies will indicate a decrease in fertility of the sample-treated group. In situations where the difference in larval activity is only marginal, the number of emerged flies per vial will be counted after another seven days. The fertilities of various dose levels are reported

only marginal, the number of emerged flies per vial will be counted after another seven days. The fertilities of various dose levels are reported for each sample. A more detailed study using a brood pattern analysis to determine germ-cells stage specificity may be used as a follow-up study if the sample shows significant reduction in fertility. Fertility studies provide information on the toxic effects of chemicals on the sperm cells of exposed adults and may be a more sensitive indicator of toxicity than lethality in the adult. The effects of chemicals on fertility can occur at any of several distinct phases including hatching of eggs, larval activity or metamorphosis.

3.4 RESULTS AND INTERPRETATION

Using the maximum applicable dose (MAD) concept, which defines the practical upper exposure limit, developed for Level 1 screening bioassays, the response of chemicals in the Drosophila melanogaster test may be ranked as high, moderate, low or not detectable. Table 3.3 gives MAD and response ranges for the Drosophila Level 1 Insect Toxicity Assay of environmental samples.

TABLE 3.3. DROSOPHILA INSECT TOXICITY ASSAY EVALUATION CRITERIA

Activity Measured	MAD[a]	Response Ranges[b]			
		High	Medium	Low	Not Detectable
Lethality (LD_{50})					
Solid Samples	50	<0.5	0.5-5	5-50	>50
Liquid Samples	500	<5	5-50	50-500	>500
Fertility (EC_{50})					
Solid Samples	50	<0.5	0.5-5	5-50	>50
Liquid Samples	500	<5	5-50	50-500	>500

[a] Assay evaluation criteria are tentative and under evaluation.
[b] Concentrations are in mg/ml for solids and slurries and µl/ml for liquid samples tested as solutions and suspensions. Insoluble liquids are reported as mg or µl per gram of yeast extract paste.

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4. Litchfield, J.T. and F. Wilcoxon. A Simplified Method of Evaluating Dose-Effect Experiments, J. Pharmac. Exp. Ther., 96:99-113, 1949.